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PHD

Control of proliferation in the yeast *Saccharomyces cerevisiae*

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Control of proliferation in the yeast

Saccharomyces cerevisiae

Submitted by Eamon Michael John McGoldrick

for the degree of Ph.D.

of the University of Bath

1986

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SUMMARY

A model has been described in which cAMP, acting through cAMP dependent protein kinase, regulates the cell cycle by the activation of key proteins. In gaining support for this model it has been shown that. A) Glucosamine, a non metabolisable glucose analogue, will reduce the population doubling time of cells metabolising glucose. B) Glucosamine's ability to act as a catabolite repressor prevents the metabolism of pyruvate. C) Glucosamine inhibits the accumulation of labelled glucose and the activity of hexokinase.

Suppressors of cdc39 were isolated using a two step procedure. From 120 glucosamine resistant colonies, 14 subsequently proved to be simultaneously temperature resistant. These fell into three groups, one of which proved to be revertants. Complementation tests demonstrated that the other suppressed strains retained a cdc39 gene. Suppression of cdc39 and glucosamine resistance segregated independently. Glucosamine resistance could not be correlated with resistance to catabolite repression.

grr1, hex2, CAT1-2<sup>d</sup> and cat2 failed to suppress either cdc28 or cdc39. hex1 did not suppress cdc28 but did indicate suppression of cdc39. This suppression may require the presence of a second gene.

Sonication, freeze/thawing and toluene permeabilization are effective methods of freeing intracellular label. Freeze/thawing was adopted as the method used in all cAMP assays. Data indicate that more sensitive assays are needed for reproducible results.



The budding index response of bcy1 cells, starved of either glucose or nitrogen, showed no significant differences to the response of a wild-type strain. Starvation for nitrogen causes a more rapid fall in viability than does starvation for glucose.

MAT heterozygosity suppressed the G1 arrest of cdc39 cells. Although ste9 was unable to suppress the G1 arrest of cdc39 cells, results indicate that ste8 might.

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DEDICATIONS

To my parents and grandparents

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MATERIALS

AND

METHODS

MATERIALS AND METHODSYEPD

- 1% Yeast Extract
- 2% Mycological Peptone
- 2% Glucose
- 2% Agar

YMM

- 0.67% Yeast Nitrogen Base (YNB)
- 4% Glucose
- 1.5% Agar

Amino acid supplements were added to give following final concentrations.

- |            |        |
|------------|--------|
| Adenine    | 20mg/l |
| Uracil     | 20mg/l |
| Histidine  | 20mg/l |
| Lysine     | 30mg/l |
| Tyrosine   | 60mg/l |
| Methionine | 20mg/l |
| Tryptophan | 20mg/l |
| Arginine   | 20mg/l |
| Leucine    | 30mg/l |

Presporulation Medium

5% Glucose  
0.5% Peptone  
0.5%  $\text{KH}_2\text{PO}_4$   
0.2%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$   
0.4% Yeast Extract  
1.5% Agar

Sporulation Medium

0.3% Sodium Acetate  
0.02% Raffinose  
1.5% Agar

pH 5.8

Tetrad Dissection Agar

1% Yeast Extract  
2% Mycological Peptone  
2% Glucose  
2% Purified Agar

Before autoclaving it was important to ensure that the agar had been fully dissolved. Plates were poured thinly and allowed to dry for several days.

67mM Phosphate Buffer

1) Prepare a 0.2M solution of  $\text{NaH}_2\text{PO}_4$ , and a 0.2M solution of  $\text{Na}_2\text{HPO}_4$ . Mix in a ratio of 51:49 then dilute 1 in 3.

Dinitrosalicylic Acid Regent

1. 10 gms of phenol are added to 22 mls of 10% NaOH. This is then made up to 1000 mls with  $\text{H}_2\text{O}$ . 6.9 gms of Sodium Bisulphite are then added to 69 mls of this solution. To this is added,

300mls of 4.5% NaOH, 255 gms of  $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 7\text{H}_2\text{O}$  (potassium sodium tartrate) and 880 mls of 1% Dinitrosalicylic acid.

#### INVERTASE ASSAY

1 ml of test solution is incubated at  $31^\circ\text{C}$  for 10 mins. 0.5% sucrose is added to start the reaction which is stopped by 1.5 ml of Dinitrosalicylic acid reagent after an appropriate time. Colour is developed by 15 mins boiling and the absorbance is read at 640 nm.

#### UPTAKE OF LABELLED GLUCOSE

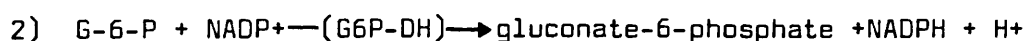
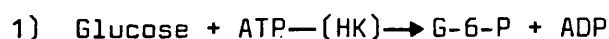
1. S. cerevisiae SR665-1 was grown at  $23^\circ\text{C}$  in YEPD to  $1 \times 10^7$  cells/ml.
2. The cells were harvested by centrifugation at 6000 rpm for 5 minutes in a Sorvall RC5B centrifuge. Following two washing cycles (spin down and resuspension in distilled water) the cells were resuspended at a density of  $2 \times 10^8$  cells/ml in <sup>20mM</sup> tris HCl buffer pH 7.6.
3. 2.5 mls of this cell suspension was added to universal bottles containing appropriate concentrations of labelled D-[1- $^{14}\text{C}$ ]glucose (Amersham International plc) and glucosamine made to a final volume of 2.5 mls with tris HCl buffer pH 6.7. This solution was equilibrated at  $30^\circ\text{C}$  in a waterbath prior to the addition of cells.
4. 1 ml samples, taken at 20 second intervals were transferred to a Millipore filter (0.45 micrometer pore size) in a Millipore multi-filter unit and washed with 5 mls of tris HCl buffer pH 6.7.
5. Filters were transferred to scintillation vials containing

7.5 mls of optiphase safe (Fisons) scintillation fluid. Radioactivity was measured in an LKB Rackbeta model 1217 liquid scintillation spectrometer.

#### ASSAY OF HEXOKINASE ACTIVITY

1. Cells of strain SR665-1 were grown to  $1 \times 10^7$  cells/ml in YEPD broth at  $23^\circ\text{C}$ . They were spun down and washed twice and finally resuspended to give a thick slurry in 0.1 M potassium dihydrogen orthophosphate buffer containing 10 mM magnesium chloride.
2. 0.7 mls of the cell slurry was mixed with 450-500 micrometer glass beads to give a thick paste in 1.5 ml Eppendorf tubes.
3. The tubes were then vortexed for three 20 second periods standing on ice between each period. The supernatant was spun from the tubes by puncturing the bottom of each tube and standing it in a second Eppendorf tube that had had its cap removed. This piggyback arrangement was then spun in a 50 ml centrifuge tube.
4. The soluble portion was decanted from the small amount of insoluble matter that passed from upper to lower tube. This portion represents a crude cell free extract. The 0.5 ml obtained from several tubes was mixed to give a single sample of several mls.
5. 1 ml of this crude extract was partially purified by passage through a Sephadex (G-50 fine, Pharmacia) column and eluted with 0.1M potassium dihydrogen orthophosphate buffer containing 14 mM magnesium chloride. This step removed small contaminating metabolites that interfered with the subsequent hexokinase assay.
6. At this point the protein content of the partially purified extract was assayed using the Bio-Rad assay kit.

7. The assay of hexokinase activity was carried out by the method of Lobo and Maitra (1977). The assay uses a coupled reaction to determine the rate of production of NADPH using excess reagents to ensure that its production is directly determined by the phosphorylation of glucose by hexokinase.



8. 0.1 ml of extract was added to a cuvette containing 0.1 ml 20mM ATP (adenosine triphosphate), 0.1 ml 2mM NADP (nicotinamide adenine dinucleotide phosphate, 0.6 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) in 0.1 ml 0.1M potassium dihydrogen orthophosphate buffer containing 10 mM magnesium chloride. The volume was made up to 2 mls by the addition of 1.5 mls of buffer.

9. The production of NADPH was assayed spectrophotometrically at 365nm. The concentration was determined by comparison with a NADPH calibration curve.

#### PROTEIN CONTENT ESTIMATION

The protein content of cell free extracts was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, West Germany), a method based on the dye binding technique of Bradford (1976). The amount of protein was calculated by comparison with a bovine serum albumin calibration curve. 0.1 ml of sample was added to a 1:4 dilution of the Bio-Rad reagent, after 5 minutes the sample was read at 595nm in a spectrophotometer.

#### MUTAGENESIS AND SELECTION FOR GLUCOSAMINE RESISTANCE

Mutagenesis to glucosamine resistance was carried out as follows:

1) SR665-1 cells were grown to a density of approximately  $5 \times 10^6$



cells/ml on a YEP+2% sodium pyruvate medium. The incubation was carried out in a 1 litre conical flask shaking vigorously in a water bath at 23°C.

2) The culture was divided to give 10 x 20mls in screw top 50 ml centrifuge tubes. 2 mls of an appropriate dilution of the stock Methanesulfonic acid ethyl ester solution (EMS) (0.5 mls of neat EMS into 5.5 mls of sterile water) was added to these tubes. The concentration range was from a 1:10 dilution down to a 1:2000 dilution. A control tube lacking EMS was also incubated.

3) These tubes were incubated in a shaking water bath at 23°C for 1 hour.

4) EMS was removed by centrifuging and discarding the supernatant in a fume cupboard.

5) The pellet in each tube was resuspended in 10mls of sterile water. Following serial dilution samples from each tube were plated onto appropriate selective media.

6) The plates were sealed with parafilm and incubated in sealed gas jars in a 23°C incubator.

7) After 14 days the plates were opened in a fume cupboard and appropriate colonies selected for characterisation. When selecting for temperature resistant colonies all growth was carried out in YEPD broth or on YEPD plates.

### TETRAD DISSECTION

- 1) A loopful of sporulating yeast culture was transferred to a drop of Beta-glucuronidase in a petri-dish. The dish is left at 30°C for approximately thirty minutes to allow digestion of the ascus wall. Microscopically this stage was reached when the ascus wall became transparent.
- 2) The digested suspension of asci was spread thinly over an arc approximately 25% the area of a Petri-dish containing specially prepared YEPD agar.
- 3) Small glass loops were made from glass rods 12-15 cms long and 3.5 mm in diameter using the De Fonbrune micro-forge. These loops were positioned in a De Fonbrune micromanipulator.
- 4) Observation was carried out using a 30x magnification, long working distance objective, mounted in a Wild M20 microscope. With a 10x magnification eyepiece, optimum resolution was achieved by using condensor settings appropriate for viewing under a 10x phase contrast objective.
- 5) The principle of tetrad dissection using a micromanipulator was that a single yeast ascus was picked from the surface of an agar plate into a glass loop about 5 times its diameter. The loop was used to move the ascus to a designated point on the agar plate. After liberating a single spore, the remaining 3 were picked and the process repeated until all four asci had been positioned.
- 6) Dissection plates containing about 12 dissected asci were sealed and incubated until colonies appeared. At this point the colonies were transferred to YEPD and subsequently tested as appropriate.

### PARTICLE COUNTING

Samples were sonicated for ten seconds at full power, in an MSE 100 watt Ultrasonic Disintegrator. Particle counting was carried out using samples diluted in azide buffer, on a Particle Data Electrozone Celloscope LTS 111 (Cheltenham, Gloucestershire)

### <sup>14</sup>C HISTIDINE LABELLING

Radioactive histidine was used to label cells in experiments designed to test the efficiency of various permeabilising methods. Loading was carried out as follows.

- 1) 200 mls of cells were grown to mid exponential phase on YEPD, filtered on a 0.45 µm filter, washed and resuspended in 50 mls of solution A (100mM citrate buffer pH 4.5 & 100mM glucose).
- 2) Cells were allowed to equilibrate at 23°C for 10 minutes, before the addition of 0.5 mls of 50mM histidine and 0.2 mls of labelled histidine.
- 3) After 10 minutes incubation with histidine, the cells were washed twice with 50 mls of citrate/glucose/histidine buffer and finally resuspended in 5.5 mls of 5mM EDTA solution.

### PREPARATION OF SAMPLES FOR cAMP ASSAYS

- 1) Samples were prepared from 20 ml of exponentially growing cells.
- 2) The cells were harvested by centrifugation and resuspended in 0.6 mls of 10% trichloroacetic acid (TCA) in 1.5 ml Eppendorf tubes.
- 3) Tubes were then plunged into liquid nitrogen for 25 seconds. Rapid thawing was carried out by immersion into hot water. This step was repeated three times.

- 4) Following centrifugation the supernatant was removed using a small syringe to determine the volume.
- 5) 0.1 ml of 1M HCl and 2 volumes of ether were added.
- 6) After a period of vigorous shaking the ether layer was removed using a thin capillary tube attached to a water pump.
- 7) Steps 5 and 6 were repeated three times to ensure complete removal of all TCA.
- 8) Ether was allowed to evaporate prior to the cAMP assay.

#### cAMP ASSAY

cAMP assays were carried out using the Amersham cAMP assay kit. The principle of the assay is that a known concentration of labelled cAMP is allowed to compete with an unknown sample for a fixed amount of cAMP binding protein. Free cyclic AMP is separated from bound cAMP by the charcoal precipitation method (Brown, Albano, Ekins, Sgherzi and Tampion, 1971).

The following procedure is taken from the product information booklet that accompanies the assay kit.

- 1) Place 14 assay tubes, plus additional tubes for unknowns into an ice bath.
- 2) The intended contents of these tubes are shown in table 5.1.
- 3) The calculation of the unknown cAMP levels is as follows. A) Blank counts per minute (cpm) are determined by averaging the cpm from tubes 1 and 2. B) Subtract the blank cpm from the average of tubes 3 and 4 to give the cpm in the absence of unlabelled cAMP (Co). C) Subtract the blank cpm from the average of duplicates from tubes 5-14 to give the cpm in the presence of unlabelled cAMP (Cx). D) Calculate Co/Cx for all

standards and unknowns. Plot the standards on linear graph paper and use this calibration curve to determine the cAMP level that gives a  $C_o/C_x$  ratio of 2 (m). This "m" value is then substituted into the following equation to give the concentration of cAMP at other  $C_o/C_x$  ratios (X).  $X = m[(C_o/C_x) - 1] \text{ pmol}$ .

#### SOURCE OF CHEMICALS

General chemicals and sugars were obtained from BDH Chemicals Ltd., Poole, Dorset. Yeast extract, Agar and Myco Peptone from Lab M, Bury, Lancashire. Beta-glucuronidase, Amino acids and IBMX were from Sigma Chemical Company Ltd, Poole, Dorset. All radioactive compounds were obtained from Amersham International plc., Buckinghamshire.

INTRODUCTION

### INTRODUCTION

How does the cell regulate its cell cycle? Over the past decade the yeast Saccharomyces cerevisiae has increasingly been used as a eukaryotic model in which to study this intriguing question. Reproduction by budding, as in yeast, provides a useful visual indicator of cell cycle status. Once a cell has reached a critical size, bud initiation occurs and subsequent cell volume increase mostly takes place in the expanding bud. Consequently, the size of the bud indicates the position in the cell cycle. The ability of yeast to grow mitotically as a haploid or diploid and to alternate between these states via karyogamy and meiosis, makes it an excellent candidate for genetic analysis. Recessive mutations can be readily selected in haploid strains and complementation analysis can then be carried out in diploids.

The use of these techniques has allowed the selection of mutants that are defective in various aspects of cell division (Hartwell, Culotti and Reid, 1970). These cell division cycle (cdc) mutations were defined by Simchen (1978) as "mutations that under non-permissive conditions alter the cell cycle in a unique way". They have allowed a cell cycle map to be produced (Hartwell, Culotti, Pringle and Reid, 1974; Figure I.1) which describes the relationship between those events leading to the production of a new bud and those involved in replication of DNA synthesis and subsequent nuclear division. By the interpretation of Hartwell et al. (1974), the yeast cell cycle is analogous to that of most other eukaryotic organisms consisting of the following periods. G1: This period constitutes the gap between

the end of mitosis and the initiation of the next DNA synthesis. Regulatory mechanisms ensure that cells remain in this period until the requirements needed for the initiation of a new cycle are met. S: DNA synthesis is initiated. G2: The cell prepares for the initiation of mitosis by moving its nucleus to the neck of the bud. M: Genetic material is partitioned into mother and daughter cells. Following this, new cell wall deposition divides the cytoplasm of the mother and daughter and the cells separate.

However, the observations that, A) the mitotic spindle forms early in the cell cycle (Pringle and Hartwell, 1981) and B) chromosome condensation is not observed (Byers, 1981), have allowed an alternative explanation (Nurse, 1985; Figure I.1).

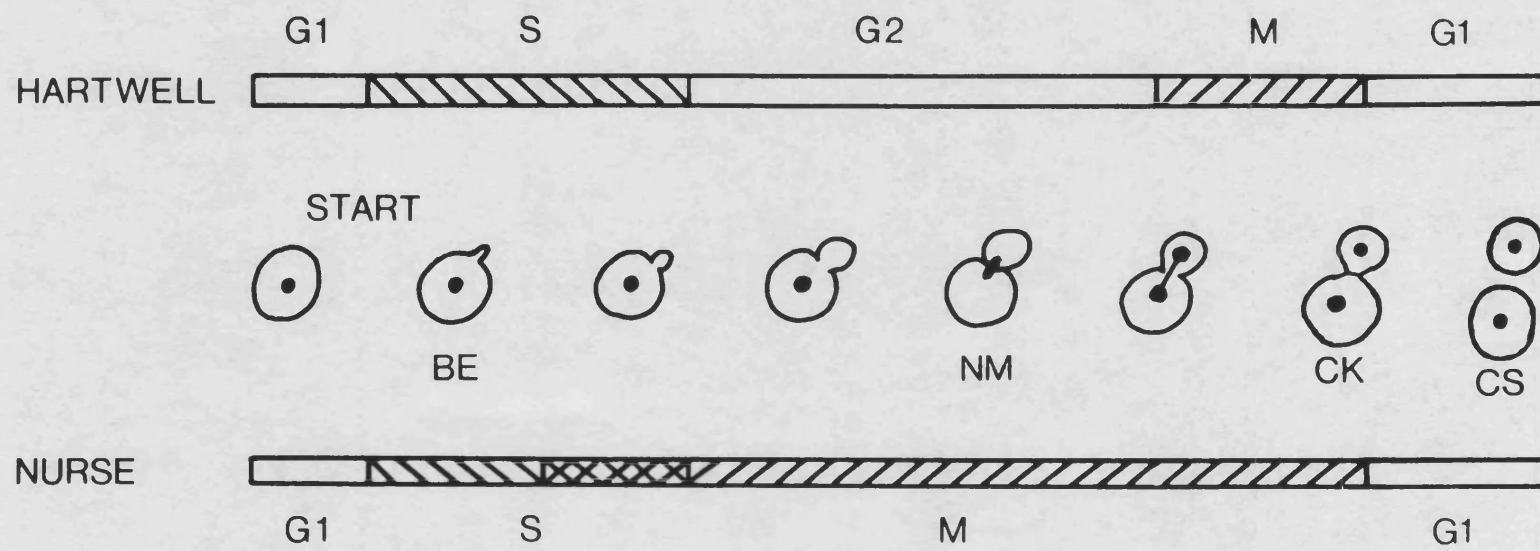
The basic difference between the two explanations concerns the timing of the initiation of mitosis. Nurse has suggested that mitosis is usually initiated prior to cell division and, since bud formation can be considered to be the first event of cell division, mitosis must begin around the time of bud initiation.

This alternative view may indicate that although Saccharomyces cerevisiae has been used as a model eukaryote, extrapolating results to other eukaryotes must be carried out with caution. Nevertheless, the observation that CDC28, a gene regulating entry into mitosis in Saccharomyces cerevisiae, is functionally homologous to CDC2, a gene controlling cell development in Schizosaccharomyces pombe (Beach, Durkacz and Nurse, 1982), indicates that events in this region of the cycle may be conserved in other eukaryotes.



FIGURE I.1     THE CELL CYCLE OF Saccharomyces cerevisiae

Landmark events of the cell cycle are described in the central section of this figure. Start indicates the point at which cells become committed to a new mitotic cycle. BE, bud emergence; NM, nuclear migration; CK, cytokinesis; CS, cell separation. Above and below are alternative interpretations of the relationship between the events of nuclear division and budding. G1 and G2 = gaps 1 and 2; S= DNA synthesis; M= mitosis. (modified after: Hartwell et al., 1974; Nurse, 1985)



CDC28 is a member of a group of genes that define "start" (Hartwell et al., 1974), the major regulatory point in the cell cycle. "Start" is the term given to the phenomenon of decision making that each cell makes before entering one of the developmental pathways open to it. The factors affecting the process of "start" are cell size, the mating pheromones secreted by one cell type that prepare the other cell type for mating and nutrient supply. The status of these allows the cell to continue a further mitotic cycle or forces it to arrest in G1. The diploid cell depends upon the same parameters in the decision to enter the meiotic cycle that precedes sporulation, and the haploid spore to move from a dormant to vegetative state (Figure 1.2). Clearly the cell needs to assay these factors. Although it is probable that the state of the environment is transmitted to the "start" system via the changes in the concentration or nature of some internal indicators, the nature of the assay or even the molecules involved are largely unknown.

Several genes known to be involved in regulation at start have been partially characterised. CDC28, CDC36, CDC37 and CDC39 have been cloned, giving translation products with molecular weights of 33 KDa, 20 KDa, 50 KDa and 100 KDa respectively (Reed, 1984). It has been demonstrated that CDC28 shows significant sequence homology with bovine cAMP dependent protein kinase, other members of the vertebrate protein kinase family, and viral oncogenes showing protein kinase activity (Lorincz and Reed, 1983). Use of an anti-CDC28 antibody co-precipitates a 40KDa protein that is phosphorylated in a reaction that is dependent

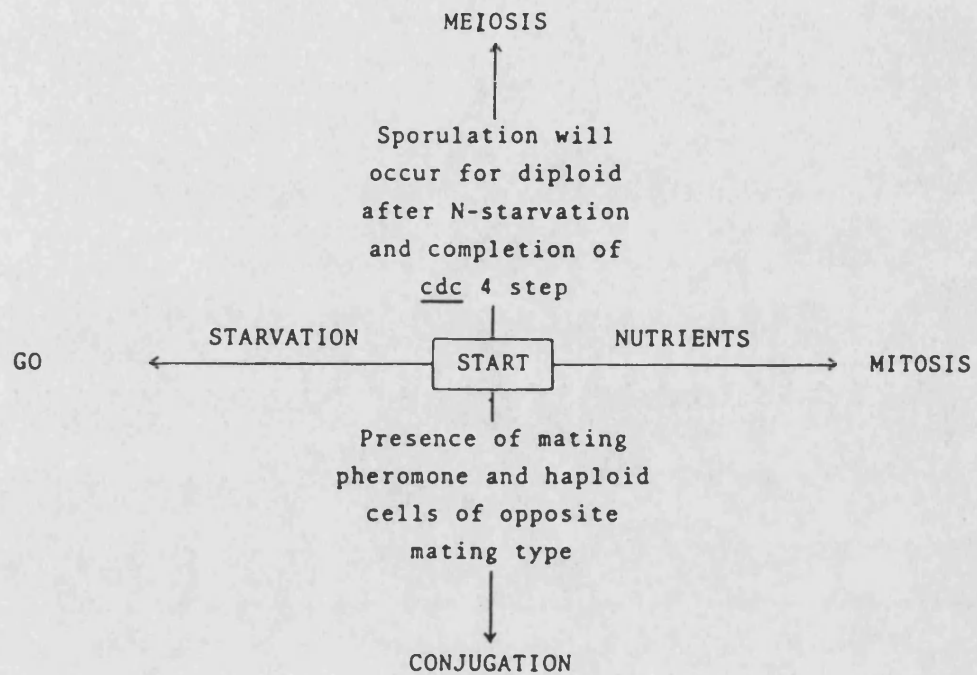


FIGURE I.2 DEVELOPMENTAL PATHWAYS FROM START

Start represents the major regulatory event in the cell cycle of *Saccharomyces cerevisiae*. At this point the cell assesses environmental factors and responds to them by entering one of the alternative developmental pathways illustrated.

upon ATP (Reed, Ferguson, Hadwiger, Ho, Lorincz, Peterson, Richardson and Wittenberg, 1985). CDC36 has regions of sequence homology with ets (transformation specific sequence of avian erythroblastosis virus E26). Although the biological function of CDC36 and all other start genes remains unknown, these observations support the suggestion that the mechanisms controlling cell proliferation in yeast and higher organism may be similar.

Since "start" represents a eukaryotic regulatory event in an organism that is amenable to genetic and molecular analysis (Roman, 1981), elucidation of cell regulation in yeast may provide data applicable to higher eukaryotes. This approach has already demonstrated that mammalian ras genes are functionally homologous to those found in yeast. Examination of yeast RAS genes has indicated them to be closely involved in cAMP metabolism (Wheals, 1985).

Cyclic adenosine 3',5'-monophosphate (cAMP) seems to have important regulatory roles in cellular systems as diverse as mammalian cells and bacteria. In Escherichia coli, this molecule, in association with a binding protein, is responsible for the initiation of transcription of proteins subject to catabolite repression (Pastan and Adhya, 1976). It has been demonstrated that cAMP plays a major role in a cascade system modulating hormonal response in mammalian cells (Robinson, Butcher, and Sutherland, 1971.).

The role of cAMP in the cell cycle of Saccharomyces cerevisiae has recently been the subject of great interest.

Evidence has been obtained that links cAMP to both pheromonal response and catabolite repression. We have investigated the evidence in great detail and a model has been proposed in which cAMP controls progression through "start" via the activation of "target" proteins by cAMP dependent protein kinase (Chapter 1). This model makes predictions concerning possible interactions between the various gene systems involved in cAMP metabolism, catabolite repression and "start".

Cdc39 is a "start" mutation that leads to a G1 arrest characteristic of cells arrested by mating pheromone (Reed, 1980). The remainder of this project is concerned with testing some of these predictions and with the elucidation of the mechanism by which CDC39 acts to co-ordinate growth and proliferation in Saccharomyces cerevisiae. Evidence suggesting a link between cdc39, the mating system and carbon metabolism is examined in chapters 3, 4 and 6. The approach taken for most of this work was to examine the interaction of various mutations by producing double mutant recombinant haploids. Chapter 2 examines the interaction between glucose and glucosamine and describes how this can be used to modulate growth rate without affecting the cell's repression status.

The approach taken in writing this thesis is, where possible, to make each section a discrete piece of work. In doing this it has become apparent that independence could only be achieved by repeating certain key elements of the literature.

CHAPTER 1  
cAMP AND THE GROWTH  
AND DEVELOPMENT OF  
SACCHAROMYCES CEREVISIAE:  
A REVIEW

cAMP AND THE GROWTH AND DEVELOPMENT OF *Saccharomyces cerevisiae*INTRODUCTION.

The ubiquitous molecule cyclic 3'-5' adenosine monophosphate (cAMP) has been ascribed a multitude of regulatory roles in both prokaryotes and eukaryotes. In some cases its involvement is unequivocal, in others contradictory data make proposed regulatory functions controversial. It is the purpose of this review to discuss the data concerning the role of cAMP in the regulation of the cellular development (mitotic cycle, meiosis and sporulation, mating, G1 arrest) of the budding yeast *Saccharomyces cerevisiae*. It includes an assessment of its relationship with catabolite repression, a physiologically adaptive process that affects cell development. An attempt has been made to explain how defects in cAMP metabolism result in aberrant cell development. It has become clear that much of the data discussed can be assigned a position in a model proposing that all cAMP effects are mediated by changes in the activity of cAMP dependent protein kinase, and that reduced phosphorylating potential is sufficient to prevent progression through G1. The "High/go low/stop" model is a collation of many observations and a demonstration that they can be placed in a logical manner on a framework built around cAMP metabolism. Such a review is particularly timely since it has been found that yeast contains genes functionally homologous to mammalian oncogenes and that some of these genes are crucially involved in cAMP metabolism. This emphasises the fact that the results from yeast may be widely applicable to other eukaryotic organisms.



### cAMP AND CATABOLITE REPRESSION

Rapidly fluctuating natural environments ensure that organisms evolve mechanisms enabling them to use available substrates to maximum efficiency. The ability to regulate the synthesis and activity of non-essential gene products can result in energetic savings that confer growth advantages, thus increasing competitiveness. Catabolite repression is an example of such a process. The presence of a fermentable carbon source allows the cell to switch off production of enzymes involved in the catabolism of less energetic substrates. The continuous processes of growth, that result in increased cell mass, and the discrete events of the cell cycle, that lead to division, are normally tightly co-ordinated (Johnston, Pringle and Hartwell, 1977). Catabolite repression plays a major role in regulating carbon metabolism, a process central to growth, and evidence has been provided that links it to cell cycle regulation (Shuster, 1982b; Ruiz, Villanueva and Rodriguez, 1984). Consequently, catabolite repression, or aspects of it, may form part of the mechanism by which cells regulate their cell cycle with respect to general metabolism.

The "glucose effect" is far reaching and rapid. It acts at translational, transcriptional and post transcriptional levels (Boker-Schmitt, Francisci and Schweyen, 1982), on processes as diverse as mitochondrial biogenesis, gluconeogenesis, the glyoxylate cycle and the TCA cycle (Polakis and Bartley, 1965; Polakis, Bartley and Meek, 1965). Catabolite repression is a feature of the glucose effect that has obvious energy conserving

functions through its ability to prevent the wasteful synthesis of numerous catabolic enzymes. The role of catabolite inactivation, a second aspect of the glucose effect, is as yet unclear. Its effects are more rapid than those of catabolite repression leading, as its name implies, to the proteolytic inactivation of several enzymes (Baker-Schmitt et al., 1982; Holzer, 1976). Once again its main purpose seems to be the conservation of energy. The inactivation of fructose 1,6,-bisphosphatase and phosphoenol pyruvate carboxykinase prevents the futile hydrolysis of ATP. Inactivation seems to be a process that has evolved to remove quickly the activity of enzymes whose actions would be deleterious if left to the growth dependent, dilution effect of catabolite repression.

Although it is clear that a fermentable carbon source, notably glucose, is the trigger for catabolite repression, the metabolic effector of catabolite repression remains unknown. The process of catabolite repression, if not the details, is sufficiently important to cross the prokaryote/eukaryote boundary. In Escherichia coli the pleiotropic effects of catabolite repression are mediated by cAMP in association with a cAMP binding protein (Ullmann and Danchin, 1983). Evidence that cAMP plays a role in catabolite repression in Saccharomyces cerevisiae was sought and the initial data indicated that intracellular cAMP varied in a manner consistent with its proposed pivotal role.

Mahler and Lin (1979) demonstrated that cAMP could overcome the glucose repression of delta-aminolevulinate dehydratase and

that the concentration needed was directly dependent upon the concentration of glucose in the growth medium. The kinetics of cAMP induced derepression of NADH cytochrome c reductase were found to compare favourably with that shown upon transfer to an ethanol growth medium. These workers determined that cells were sufficiently permeable to exogenously supplied cAMP to mimic the rise in cAMP that they had seen upon transfer from repressive to derepressive medium. Using labelled cAMP they demonstrated that cells were accumulating cAMP at a rate sufficient to account for the change accompanying derepression.

Several groups have indicated that the intracellular concentration of cAMP increases as the medium becomes less repressive. Schamhart, Berge and van de Poll (1975) found 80% more intracellular cAMP in galactose grown cells than those grown on glucose. Montenencourt, Kuo and Lampen (1973) indicated a 20% increase in the intracellular concentration of cAMP of cells using low glucose over those using high glucose. Using Saccharomyces fragilis, Sy and Richter (1972b) measured a two fold increase in cAMP when cells were using lactate rather than glucose. Although the measured cAMP levels differ between the groups, all concur about the relationship between cAMP and catabolite repression. However, these results are in stark contrast to those obtained by Eraso and Gancedo (1984) who claim that "the lowest levels of cAMP are found in yeasts not subject to catabolite repression". This difference of opinion highlights the difficulty of cAMP assays. Low levels of cAMP, enzymic degradation and interference by contaminating metabolites demand

that cAMP assays are carried out with great care. Eraso and Gancedo suggest that the earlier conflicting data are "artifacts or difficult to explain" and propose that "most measurement problems are caused through inaccuracy" (Eraso and Gancedo, 1984).

The ability of cAMP to overcome glucose repression has been shown using alpha-glucosidase (Wiseman and Lim 1977); aminolevulinate synthetase, aminolevulinate dehydratase, malate dehydrogenase, cytochrome c oxidase and NADH cytochrome c reductase (Mahler and Lin, 1979); alcohol dehydrogenase (Schlanderer and Dellweg, 1974), and mitochondrial biogenesis (Fang and Butow, 1970). Tsuboi, Kamisaka and Yanagishima (1972) have demonstrated that exogenous cAMP can partially relieve the repression of sporulation by glucose. Any increase in cAMP accompanying a shift to derepressive conditions would involve a change in the activity of enzymes regulating intracellular cAMP levels. Jaynes, McDonough and Mahler (1982) found that the activity of adenylate cyclase, the enzyme that synthesises cAMP, is elevated in non-repressive medium.

Stimulating adenylate cyclase or inhibiting cAMP phosphodiesterase (which degrades cAMP) should increase the intracellular concentration of cAMP mimicking the effects of exogenously supplied cAMP and circumventing the problems of impermeability to cAMP. This approach has been used on several occasions to investigate putative cAMP effects, and has produced conflicting results. Mahler and Lin (1979) showed that sodium fluoride (NaF), a proposed stimulator of adenylate cyclase was

effective at derepressing aminolevulinate dehydratase, but that theophylline, a phosphodiesterase inhibitor, had no effect on release from repression. Wiseman and Lim (1977) found that 10mM NaF could cause a 40% stimulation of adenylate cyclase and that 1% NaF would release the glucose repression of alpha-glucosidase in intact yeast cells. In contrast, Casperson, Walker, Brasier and Bourne (1983), Liao and Thorner (1980) and Jaynes et al. (1982) failed to find any stimulatory effect of the fluoride ion on adenylate cyclase. Inhibition of phosphodiesterase by theophylline reduced the glucose repression of sporulation (Tsuboi and Yanagishima, 1973) but was unable to mimic the effects of exogenous cAMP on glucose inactivation (Tortora, Burlini, Hanozet and Guerriore, 1982). The evidence thus presented generally supports the view that any relationship between catabolite repression and cAMP is a negative one such that high cAMP is associated with derepression. Evidence conflicting with this is provided by Tortora et al. (1982) who state that "there was a strong negative correlation between cAMP levels and the extent of inactivation". Since catabolite repression and catabolite inactivation respond to the same initial signal (glucose), it is unlikely that cAMP could be correlated positively with a release from repression and negatively with a release from inactivation.

#### Mutations Affecting cAMP Metabolism or Catabolite Repression

Additional evidence concerning the relationship between cAMP, catabolite repression and the cell cycle is provided by a study of the characteristics of catabolite repression and cAMP

metabolism mutants. If the effects of catabolite repression are modulated via changes in the internal concentration of cAMP, mutants resistant to catabolite repression might be expected to have high levels of cAMP in the presence of high concentrations of glucose. Montenencourt et al., (1973) isolated the first catabolite derepression resistant mutants and failed to find any correlation between invertase activity and cAMP concentrations. The flk1 mutant (Schamhart et al., 1975) has between 15-20% more cAMP than its parent but its cAMP levels still fluctuate like wild type in response to catabolite repression. Michels and Romanski (1980) indicate that their qlr1 strain does not have significantly altered levels of adenylate cyclase and phosphodiesterase. Although it is clear that in these mutants cAMP levels do not change as expected were cAMP the modulator of catabolite repression, it is possible that the genetic lesions of the strains are "downstream" from cAMP. Consequently, cAMP could still be affected by the carbon source but is unable to transduce this into derepression because of mutational insensitivity to cAMP.

A second set of mutants, isolated by Matsumoto, Uno, Oshima and Ishikawa (1982a) provides evidence that makes a direct role for cAMP in catabolite repression induction unlikely. These mutants do however, have characteristics that suggest that the "glucose effect" could involve cAMP indirectly. They reasoned that if high cAMP prevented catabolite repression, cells capable of taking up enough to use as an adenine source would have cytoplasmic concentrations high enough to prevent repression of

galactokinase (Matsumoto, Uno, Toh-e, Ishikawa and Oshima, 1982b). Using mutant strains capable of this they demonstrated that the synthesis of galactokinase was still subject to catabolite repression, but that galactokinase synthesis was stimulated by exogenous cAMP. However, if cAMP were involved in galactokinase synthesis one would predict that cells incapable of producing cAMP would be abnormal in galactokinase synthesis. Evidence was provided that galactokinase synthesis is normal in strains lacking detectable cAMP (Matsumoto, Uno, Ishikawa and Oshima 1983c).

In a second paper the argument was taken one step further by the prediction that cells completely lacking cAMP, should be defective in the synthesis of enzymes subject to catabolite repression. Loss of adenylate cyclase function (cyr1) can be suppressed by bcy1, a mutation that leads to the production of a protein kinase that has lost its dependency on cAMP. Cells containing these mutations allow an examination of all cAMP mediated effects with differentiation between those dependent and independent of protein kinase. Using these, they found that cells lacking cAMP (cyr1, or cyr1/bcy1 strains) showed similar levels of galactokinase and alpha-D-glucosidase activity to wild type under inductive and non-inductive conditions. These results imply that cAMP is not involved in catabolite repression of these two enzymes.

It is not possible to exclude a role for cAMP in the catabolite repression of enzymes other than invertase and galactokinase. Support for this view comes from several

quarters: i) Invertase activity is significantly higher in cells lacking the regulation of protein kinase by cAMP (Matsumoto et al., 1983c); ii) The levels of invertase and alpha-D-glucosidase (both repressible enzymes) measured in a cyr1 strain under derepression conditions were found to be about 5% of the wild type level (Matsumoto, Uno and Ishikawa 1984). It was concluded that, "From these results cAMP is implicated to be involved in the derepression of these enzymes". An explanation was offered for a role for cAMP dependent protein kinase in the modification of chromatin to allow derepression of specific enzyme synthesis. iii) The regulation of catabolite repression is via a complex, multigenic system, and although the initial trigger for the repression of enzymes is common to all, the details of their regulation may involve quite different mechanisms. This is best illustrated by the range of enzymes that the many catabolite derepression resistant mutants affect (Table 1), causing Entian to comment, "All mutants so far obtained showed pleiotropic regulatory defects. Analysis of interaction of these genes is complicated by the fact that different repressible enzymes are under the regulatory control of overlapping, but not identical sets of genes" (Entian and Zimmermann, 1982). Thus, it was found, (Matsumoto et al., 1983c) that although bcy1 had no effect on the derepression of galactokinase or alpha-D-glucosidase, it did affect invertase. However, these enzymes would be placed in the same group by the hex1, hex2 (Entian 1980, Entian and Mecke 1982, Entian and Zimmermann 1980), grr1 (Bailey and Woodward 1984) and glr1



TABLE 1.1

The range of enzymes affected by various catabolite repression mutations and mutations in cAMP metabolism.

	67	3	53	40	40	21	21	21	54	93	93	93	12	12	51	50	REFERENCE
	<u>flk</u>	<u>grr1</u>	<u>glr1</u>	<u>hxx1</u>	<u>hxx2</u>	<u>hex1</u>	<u>hex2</u>	<u>cat80</u>	<u>FH4C</u>	<u>cat2</u>	<u>CAT1</u>	<u>cat1</u>	<u>ccr2&amp;3</u>	<u>ccr1</u>	<u>cyr1</u>	<u>bcy1</u>	MUTATION
									A	B	B	B	C	C	O	O	
Methyl Glucosidase	-																
Invertase	-	-				-	-	-	-			o			-	+	
Alpha D Glucosidase	-	-	-			-	-	-	-	-	-	+			-	o	
Galactokinase		-	-													o	
Acid Phosphatase															-	+	
Fructose 1,-6, Bisphosphatase						o	o	o		-	-	+	+	+	-		
Hexokinase		+		-	-	-	+	o									
Galactosidase			-														
Succinate Dehydrogenase	-					-	-	o					+	+			
Cytochrome c-Oxidase		-	-														
Isocitrate Lyase						o	o	o		-	-	+	+	+			
NADH Dehydrogenase						-	-	c									
Malate Synthase						o	o	o					+				
Cytochrome c Reductase			-														
Malate Dehydrogenase						-	-	-		-	-	+	+	+	-		

- = -ve EFFECT      o = NO EFFECT  
+ = +ve EFFECT      Blank = NO DATA

A= Strain Name      C= Unable To Derepress  
B= Regulatory Mutants      O= cAMP Metabolism Mutants

(Michels, Hahmengerger and Sylvestre 1983) mutations. Further evidence of an involvement for cAMP in the "glucose effect" is provided by Tortora, Burlini, Leoni and Guerriore (1983). Using a cyr1 strain, they demonstrated that mutants lacking cAMP are deficient in the glucose inactivation of fructose-1,6-bisphosphatase, cytoplasmic malate dehydrogenase, and phosphoenolpyruvate carboxykinase. Proposing that, the involvement of a cAMP dependent protein kinase in the mechanism of the cyclic nucleotide-triggered inactivation, is plausible. Although this evidence supports an involvement for cAMP in the "glucose effect" it is in direct conflict with that of Matsumoto et al. Tortora et al. indicate that high cAMP levels would trigger facets of the "glucose effect" yet Matsumoto et al. would argue that high cAMP levels suppress other aspects.

#### Mechanism of Catabolite Repression

As yet the mechanism of catabolite repression is unknown. Recent work by Entian, Kopetzki, Frohlick and Mecke (1984), and Entian and Frohlick (1984) reveals the importance of hexokinase isoenzyme PII in catabolite repression. They propose that it has a catalytic, phosphorylating domain with a second domain acting as a trigger for catabolite repression. The altered hexokinase activity of many catabolite repression mutants corroborates their proposal (Michaels et al., 1983; Entian and Mecke, 1982; Bailey and Woodward, 1984; Lobo and Maitra, 1977b).

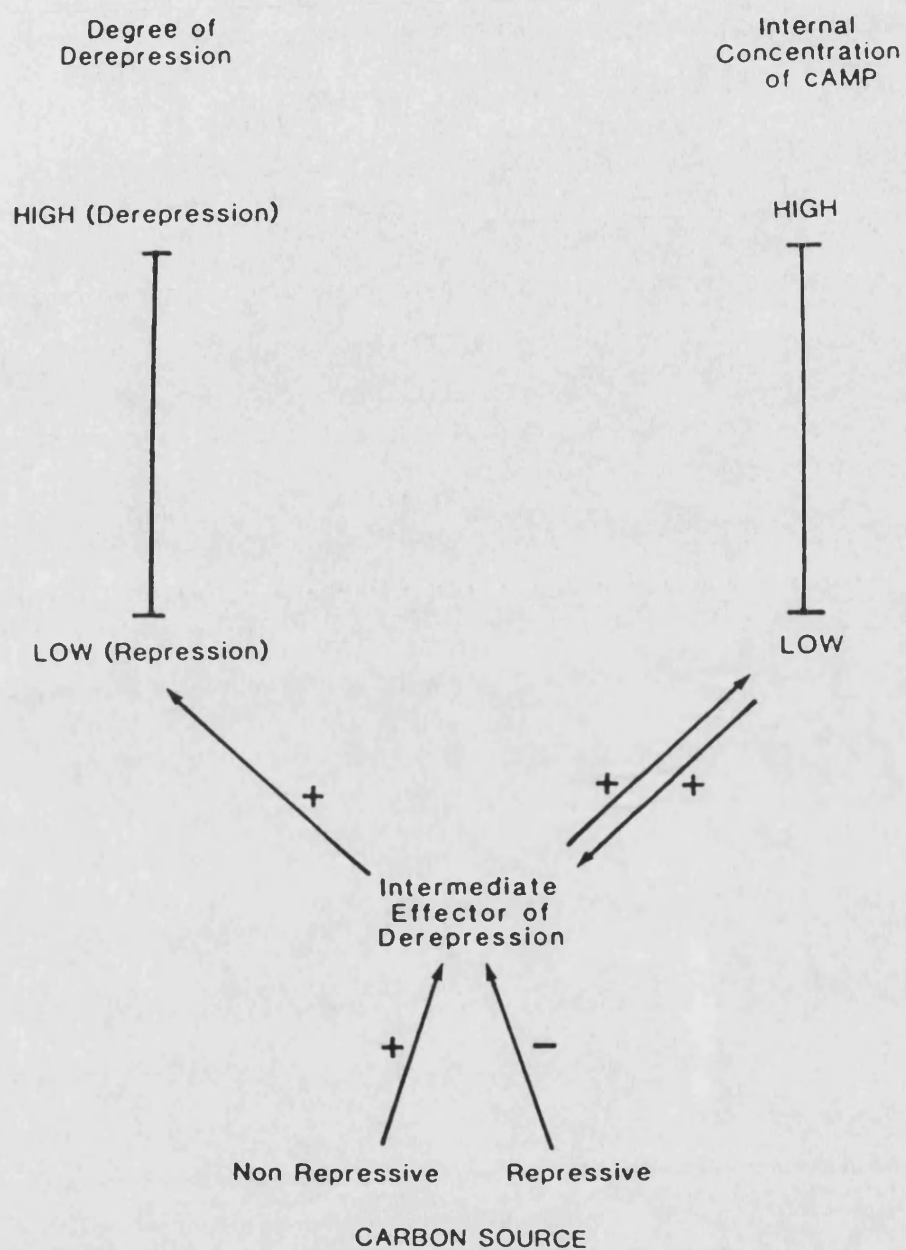
#### Conclusions

In conclusion, the evidence presented seems to exclude the possibility that cAMP mediates catabolite repression in a manner

analogous to that in E. coli. (Magasanik, 1961) It is also apparent that if intracellular levels of cAMP are related to the degree of repression, the relationship is not absolute. The results of Mahler and Lin's work (Mahler and Lin, 1979), the existence of derepression mutants with wild type fluctuations of cAMP, and the evidence suggesting that cAMP is involved in derepression can be accommodated in a single model (Figure 1.1). The basis of this model is that catabolite repression and cAMP levels are modulated by a single intermediate that is responsive to feedback control from cAMP. In this way increasing the intracellular cAMP would reduce the activity of the intermediate with a subsequent reduction in the degree of derepression. It allows the level of cAMP to rise as the cells become derepressed and, by blocking one or other of the branches, offers an explanation for catabolite repression in the absence of cAMP and fluctuations in cAMP in the absence of catabolite repression. The model calls for catabolite derepression to be an active process inhibited by glucose. Evidence that derepression is not simply the absence of repression is provided by mutants that are unable to derepress. The implication is that the wild type gene products of SNF1 (Carlson, Osmond and Botstein, 1981), CCR1, CCR2, and CCR3 (Ciriacy, 1977) are involved in an active derepression. Zimmermann, Kaufmann, Rasenberger and Haussmann (1977) isolated mutants unable to derepress by selecting for revertants of repression resistant strains. From their phenotypes, they concluded that there was genetic regulation over the kinetics of derepression, and proposed that glucose blocks an

FIGURE 1.1      MODEL RELATING cAMP AND CATABOLITE REPRESSION

A schema offered to explain elements of the data relating cAMP and catabolite repression. In this interpretation, cAMP and catabolite repression are indirectly linked via a putative intermediate. It calls for catabolite derepression to be an active process under the control of an effector whose activity is regulated by the carbon source. Repressive substrates inhibit the effector, derepressive substrates increase its activity.



+ = Stimulation  
 - = Inhibition

FIGURE 1.1 THE RELATIONSHIP BETWEEN cAMP AND  
 CATABOLITE REPRESSION.

active derepression via the action of the CAT1 and CAT2 gene products.

#### cAMP, CATABOLITE REPRESSION AND MATING

The preceding section dealt with the controversial relationship between the "glucose effect" and cAMP. This section deals with the interactions of both cAMP and the glucose effect with pheromone response in the yeast Saccharomyces cerevisiae. Expression of the MATalpha locus leads to the suppression of MATa specific genes and the activation of those specific to MATalpha cells. Alpha-factor is the product of one such alpha-specific gene, its MATa equivalent being a-factor. The effects of these two mating pheromones is to initiate events, as a prelude to mating, that lead to cell cycle arrest at a point in G1. This point has been defined as "start" and is the major regulatory event in the cell cycle (Hartwell, Culotti, Pringle and Reid, 1974). The effects of alpha-factor on MATa cells are pleiotropic. It causes increased agglutination, altered cellular morphology (shmooing), as well as cell cycle arrest (Thorner, 1981). The manner in which these changes are effected is not yet known.

Liao and Thorner have proposed that cAMP is the intracellular modulator of alpha-factor (Liao and Thorner, 1981).

In this sense cAMP would be acting as a second messenger, a role it is known to have in the hormonal response of mammalian cells. They demonstrated that exogenous cAMP could antagonise the alpha-factor arrest of intact cells (Liao and Thorner 1981) in a

manner analogous to the release of sporulation from glucose repression (Tsuboi et al., 1972). Continuing the comparison, both studies demonstrated that, in their respective systems, theophylline could mimic the effects of cAMP by its inhibitory effects on phosphodiesterase (Liao and Thorner, 1981; Tsuboi and Yanagishima, 1983). Using a labelled cAMP production assay, Liao and Thorner measured the effects of alpha-factor on adenylate cyclase from isolated membrane fragments and demonstrated inhibition. As a control, they showed that the in vitro effects of active and inactive alpha factor correlate with those in vivo.

The conclusion of their work is that alpha-factor effects are mediated by lowering the intracellular concentration of cAMP. However, since alpha-factor effects in vivo were assayed using only G1 arrest as an indicator, it is possible that alpha-factor action is more complicated than they suggest, with other effects (agglutination etc) working via transduction pathways independent of adenylate cyclase. Support for this is provided by Baffi, Shenbagamurthi, Terrance, Becker, Naider and Lipke (1984). Using alpha-factor analogues they have been able to demonstrate that two of the effects of alpha-factor can be dissociated. Certain analogues are able to induce agglutination without morphogenesis and vice versa. This would not be possible were both effects dependent upon the same transduction mechanism. Analysis of alpha-factor dose-response curves by Moore (1983) adds further support for an independent, multipathway mode of action for alpha-factor. Finding that the half maximal response for shmoo formation was two orders of magnitude higher than that of

agglutination and cell division arrest, she concluded: "These results are consistent with the same high affinity alpha-factor receptor mediating both cell division arrest and agglutination. A different system of lower affinity must mediate project (shmoo) formation." Additional evidence for the proposal of multiple pathways in alpha-factor response is provided by the observation that cAMP can antagonise cell division arrest (Liao and Thorner, 1981) but has no effect on the induction of agglutination (Moore, 1983). The results of Liao and Thorner have been questioned by Casperson et al. (1983). Whilst examining effectors of adenylate cyclase, notably GTP, they failed to demonstrate any inhibitory effects by alpha-factor.

A proposal that catabolite repression and/or cAMP are involved in mating would suggest that mutants defective in either aspect would have altered responses to mating pheromone. In the absence of exogenous cAMP, cyr1 strains arrest in G1 as non growing cells and are incompetent to mate (Matsumoto et al., 1982a). This phenotype is typical of class II start mutants that arrest because defects in growth prevent them from proliferating (Reed, 1980). An example of this class is the tra3 (renamed GCD1) mutant which is defective in the general regulation of amino acid biosynthesis (Wolfner, Yap, Messenguy and Fink, 1975). Although reciprocal shift experiments suggest that cyr1 strains arrest before the point of nutrient starvation, its continued viability, a characteristic of class II mutants, and its functional homology with and allelism to cdc35 (Mortimer and Schild, 1985), an accepted class II start mutant, place it firmly



in this group. The ability of bcy1 to suppress both cyr1 and nutrient starvation (Matsumoto, Uno and Ishikawa, 1983a) corroborates this placing. This phenotype contradicts the expected phenotype based on Liao and Thorner's work. If alpha-factor inhibits adenylate cyclase, cells lacking adenylate cyclase should arrest as alpha-factor treated cells. This phenotype is used to define the class I start mutants of which cdc28 is an example (Reid and Hartwell, 1977). The most important difference between the class I and class II mutants is that class I mutants retain the ability to grow and mate. Consequently they cannot be attributed to simple, non-specific defects in growth. Further contradictory data are provided by the pde1 and IAC mutants (Uno, Matsumoto and Ishikawa, 1983a). Both lead to elevated levels of intracellular cAMP, the former by inhibiting cAMP phosphodiesterase, the latter by producing an overactive adenylate cyclase. Neither show any alterations in their response to mating (Matsumoto et al., 1983a). Finally, one might expect that Liao and Thorner's predicted mode of action for alpha-factor would lead to a sterile phenotype in cells insensitive to cAMP regulation. This is not the case since the bcy1 mutant retained pheromone sensitivity (Matsumoto et al., 1983a). The obvious conclusion to be drawn is that the cAMP mutants exclude the possibility of alpha-factor working exclusively through cAMP. However, the inability of alpha-factor pretreatment to increase the mating efficiency of cyr1 strains led Matsumoto et al. (1983a), to conclude that "cyr1 plays an essential role in mating". Using their evidence, the CYR1 gene

product must act via cAMP to stimulate protein kinase. The mating efficiency of bcy1 strains makes improbable any protein kinase independent cAMP mediated events. This would suggest that in wild type, as the activity of cAMP dependent protein kinase falls below a threshold value, mating efficiency would begin to decline. Such a fall may be induced by factors affecting the intracellular cAMP concentration.

Lipke, Taylor and Ballou (1976), whilst studying the effects of alpha-factor on S. cerevisiae, noted that MATa cells failed to respond to alpha-factor in the absence of a carbon source and that their sensitivity was directly related to the glucose concentration. This observation was examined further by Ruiz et al. (1984), who reported a similar sensitivity to glucose and demonstrated that a concentration of alpha-factor sufficient to arrest cells growing on 2% fructose had no effect on cells using pyruvate or glycerol. By looking at the residual alpha-factor activity remaining in the medium after recovery of cells growing on various carbon sources, Ruiz et al. suggested that the decreased sensitivity of cells growing on pyruvate was due to increased endopeptidase mediated alpha-factor inactivation. These results raise the possibility that the production of endopeptidase is subject to a degree of catabolite repression. The observation that two hours incubation under derepression conditions, in which cells have recovered from alpha-factor arrest, leaves enough residual alpha-factor activity to arrest repressed cells led Ruiz et al. to state, "In any case, the higher rate of degradation of alpha-factor by MATa cells in

catabolite derepression media might not be the only reason to explain the inability of the pheromone to arrest MATa cell growth." The alternative explanation offered is that the effects of alpha-factor are triggered by a reduction in the concentration of cAMP to below a threshold level, and that it is necessary to have reductions caused by both alpha-factor and catabolite repression before the threshold can be reached. To support a link between alpha-factor and catabolite repression they showed that alpha-factor will partially inhibit the synthesis of invertase in derepressed cells. An indirect link between catabolite repression and mating is provided by the observation that the start mutations cdc36 and cdc39 (Reed, 1980) are suppressed in carbon catabolite derepression medium (Shuster, 1982b). This allowed cells containing these temperature sensitive mutations to proliferate at the restrictive temperature on a derepressive carbon source. It has subsequently been demonstrated that expression of the G1 arrest aspect of these start mutants is subject to regulation by the MAT locus (Shuster, 1982a; Connolly, Bugeja, Piggott and Carter, 1983). This has prompted the suggestion that since the alpha factor like, G1 arrest of these mutations is only seen in cells with an active, pheromone responsive, mating system, that they may be elements of the pheromone transduction pathway. Although MAT heterozygosity can suppress G1 arrest, it cannot suppress the growth defect of cdc36 strains (Shuster, 1982a).

The conclusion, that cAMP must be low to allow mating, contrasts with that of Matsumoto et al. who would predict that high levels are needed. This apparent contradiction can be resolved if an alternative explanation is offered. The claim that the CYR1 gene product, and therefore cAMP, is involved in mating is based on the observation that cyr1 cells synchronised at the alpha-factor arrest point prior to shift up do not mate at the restrictive temperature (Matsumoto et al., 1983a). Since alpha-factor arrests cells at start but allows continued growth, it is possible that the ability to mate is dependent upon the ability to grow. Consequently, non growing cyr1 strains are mating deficient. The bcy1 mutation restores the ability to grow allowing cyr1 strains to mate. In this case the observed inability of cyr1 strains to mate is the result of a failure to grow rather than direct cAMP effects.

Although the observations of Ruiz et al. (1984) lead to the prediction that cells which are permanently derepressed should have reduced mating efficiencies, there are no reports that this is the case. An explanation for this may be that mutational derepression and true catabolite derepression are not identical. Indeed, the evidence presented earlier concerning the range of enzymes affected by the various mutations would tend to support this.

In conclusion, both Matsumoto et al. and Casperson et al. provide evidence contrary to Liao and Thorner's model of cAMP involvement in mating. Although Liao and Thorner have demonstrated inhibition of adenylate cyclase in vitro, their

inability to measure intracellular cAMP levels has prevented them verifying it in vivo. The evidence makes it unlikely that cAMP is the mediator of alpha-factor effects. However, cAMP and theophylline do antagonise alpha-factor mediated effects in a manner analogous to that of derepression media. Consequently, cAMP may be indirectly related to the mating pheromone response via its effects on catabolite repression.

#### cAMP AND SPORULATION

Under conditions of nitrogen limitation, diploid yeast cells enter the meiotic cycle (Esposito and Klapholz, 1981). It has been proposed that cAMP plays a role in the decision of a diploid cell to sporulate (Matsumoto, Uno and Ishikawa, 1983b). It has been shown that the sporulating behaviour of cells mutant in cAMP metabolism is aberrant. CYR3 is a mutation that leads to a cAMP dependent protein kinase altered in such a way as to require elevated levels of cAMP for the dissociation of its catalytic and regulatory subunits. Its effect is to reduce the activity of protein kinase for a given concentration of cAMP and thus mimic reductions in cAMP for all cAMP dependent protein kinase mediated effects. CYR3 and cyr1 homozygous diploid cells sporulate in nutrient media (Matsumoto et al., 1983b). The observation that bcy1, a suppressor of both CYR3 and cyr1, is sporulation deficient corroborates the claim that cAMP has a role in sporulation. It is claimed that sporulation is dependent upon G1 arrest. Since the bcy1 mutation prevents nutrient starved G1 arrest, these cells are unable to sporulate. This proposed

relationship between G1 arrest and sporulation is supported by other mutations that lead both to the inability to G1 arrest and the inability to sporulate (eg. ard1 (Whiteway and Szostak, 1985), sra1 ( K. Tatchell, L. Robinson, J. Cannon, R. Schultz, M. Breitenbach, D. DeFeo-Jones, G. Temeles. J. Gibbs, I. Sigal and E. M. Scolnick., Abstr. J. Cell. Biochem. Supp. 9C. 1985. 1422. p.114 ), RAS2 (Toda, Uno, Ishikawa, Powers, Kataoka, Broek, Cameron, Broach, Matsumoto and Wigler, 1985) and glc1 (Ortiz, Maia, Tenan, Braz, Padrao, Mattoon and Panek, 1983)).

Although the mutants of Matsumoto et al. demonstrate a relationship between cAMP and sporulation they are unable to show if the observed effects are direct or indirect. The conclusion that "cAMP works as a negative effector on the initiation of meiosis via the activation of cAMP dependent protein kinase" (Matsumoto et al., 1983b) implies a direct role for cAMP in the regulation of sporulation. One of the greatest difficulties encountered when working with mutants like cyr1 is to differentiate between primary effects in which cAMP has a direct regulatory role and secondary effects caused by the inability of cyr1 cells to continue growth.

The involvement of cAMP and cAMP dependent protein kinase in a wide spectrum of metabolic effects is demonstrated by the highly pleiotropic nature of both cyr1 and bcy1 cells (Matsumoto et al., 1982a). The existence of mutations not implicated in cAMP metabolism that lead to similar phenotypes should serve to exemplify the caution needed in differentiating primary and secondary effects. Dawes (1975) has isolated a series of mutants

that lead to sporulation on rich media. These spd (sporulation deficient) mutants show many similarities to the cyr1 mutation of Matsumoto et al. (Dawes and Calvert, 1984; Matsumoto et al., 1982a). For both, haploids arrest in G1 as nutrient starved cells. Both show similar patterns of sporulation on media containing non-fermentable carbon sources whilst retaining sensitivity to glucose repression of sporulation (Vezinhiet, Kinnaird and Dawes, 1979). Further evidence that cyr1 and spd1 are phenotypically, though not genetically similar, is demonstrated by the remarkable similarity of phenotypes shown by suppressor mutations of these genes. Both bcy1, and the spd1 suppressing spo mutants (Calvert and Dawes, 1984), are sporulation deficient; both suppress the ability to G1 arrest under nutrient starvation; both lose viability rapidly under starvation conditions and neither shows commitment to premeiotic DNA synthesis or intragenic recombination under restrictive conditions. The phenotype of the spd1 mutation has been attributed to a defect in a "central metabolic function involving some, but not all, non-fermentable carbon sources" (Dawes, 1983), a secondary effect of which is to alter nitrogen repression of sporulation. Vezinhiet et al. (1979) state that the spd1 mutants are "probably not affected directly in events unique to the initiation of sporulation", indicating them to be secondary consequences of mutations that affect inputs to start via growth.

The physiology of spd1 and spo1 mutants leads Calvert and Dawes to suggest that they relate to the "fundamental and complex regulatory connection between respiration and sporulation"

(Calvert and Dawes, 1984). Considering the phenotypic similarities between cyr1 and spd1, and bcy1 and spo1, it is possible that the explanation offered to account for the sporulating properties of spd1 and spo1 can be extended to cyr1 and bcy1.

Rather than a direct role, the evidence that Matsumoto et al. present supports the suggestion that the altered meiotic features of the cyr1 and bcy1 cells can be explained in terms of a secondary metabolic defect leading to nitrogen starvation deregulation. Wild type diploid yeast cells undergo G1 arrest in response to a failing nutrient supply. They remain in G1 until the nitrogen supply becomes inadequate. Thus it is the lack of nitrogen rather than G1 arrest that triggers commitment to meiosis.

Cells containing the cyr1 mutation arrest as unbudded, G1 cells in the absence of cAMP. This phenotype is identical to that of cells starved for nitrogen and other nutrients (Johnston et al., 1977). Sporulation in wild type cells is subject to repression by glucose (Tsuboi et al., 1972) and nitrogen (Pinon, 1977). Matsumoto et al. have shown that cyr1 diploids will sporulate in nutrient medium with an acetate carbon source (Matsumoto et al., 1983b). The observation that sporulation is reduced at both permissive and restrictive temperatures by the presence of glucose indicates that these cells retain their sensitivity to glucose repression. Although they have examined the effects of various carbon sources on the sporulation of cyr1 diploids, there are no data on their sporulation relating to



differing nitrogen sources. Consequently, direct evidence concerning their sensitivity to nitrogen repression is not available.

Although it is claimed that the bcy1 mutation is able to suppress nutrient induced G1 arrest, the experimental design only tests the ability to suppress G1 arrest caused by nitrogen limitation (Uno, Matsumoto and Ishikawa, 1982). The dramatic reduction in viability of bcy1 diploids subject to nitrogen starvation suggests that these cells have lost the regulation of start imposed by nitrogen deficiency. They attempt to complete a second cell cycle but in the absence of nitrogen cannot continue growth, inviability being the result of the subsequent "random arrest". The phenotype of cyr1 cells can be explained if one of the effects of reduced protein kinase activity is to abolish the cells ability to assay nitrogen. In this situation cyr1 haploids would arrest as nitrogen starved cells. The diploid would, in the absence of glucose, G1 arrest and immediately enter the meiotic cycle in response to the bogus nitrogen starvation trigger. The corollary is that since bcy1 suppresses the ability to detect or respond to nitrogen limitation, bcy1 diploids would not and do not sporulate even when nitrogen is absent. We propose that cAMP and cAMP dependent protein kinase have a role in the mechanism by which the cell monitors its nutritional status. The similarity shown between the properties of cAMP metabolism mutants and the RAS mutants (discussed below), which are also proposed to be involved in nutrient response, supports this ( K. Tatchell, L. Robinson, J. Cannon, R. Schultz, M.

Brietenbach, D. DeFeo-Jones, G. Temeles. J. Gibbs, I. Sigal, and E. M. Scolnick. Abstr. J. Cell. Biochem. Supp. 9C. 1985. 1422. p.114).

Involvement of cAMP in nitrogen repression is suggested by the work of Tsuboi and Yanagishima (1973). Although their paper relates mainly to glucose repression of sporulation they stated that the repression of sporulation was observed "only in the presence of yeast extract and glucose". When the yeast extract of the presporulation medium was replaced by a mixture of vitamins, cAMP had no effect on releasing the repression of sporulation. They concluded that "yeast extract in the presporulation culture seems to play an important role in the repression of sporulating ability." Subsequently Pinon (1977) demonstrated the sensitivity of sporulation to nitrogen. Consequently Tsuboi and Yanagishima may have observed cAMP relieving the repression of sporulation caused by the nitrogen sources in yeast extract.

In an earlier paper (Tsuboi et al., 1972) it was demonstrated that cAMP could relieve the repression of sporulation by glucose in systems not complicated by the presence of yeast extract. This result would suggest an involvement of cAMP in glucose repression of sporulation. However, the retention of glucose sensitivity by cyr1 mutants and the recent finding that fluctuations in cGMP and GTP but not cAMP are necessary for meiosis ( Z. Olempska-Beer. Abstr. J. Cell. Biochem. Supp. 9C. 1985. 1411. p.110) are contrary to this. Matsumoto et al. indicate that low cAMP is needed for sporulation yet Tsuboi

et al. imply that high cAMP would be required. Although the nature of the relationship is unclear, both groups agree that cAMP is involved in regulating the switch from mitotic to meiotic cycles.

#### cAMP AND ONCOGENESIS

The proposition that cAMP is involved in cell cycle regulation is supported by the properties of mutants defective in cAMP metabolism (Matsumoto et al., 1983b). Although the primary defect of the bcy1 mutation is known, the reasons for its resultant phenotype are not clear. The observation that bcy1 fails to respond to nutrient limitation, and the inability of homozygous bcy1 diploids to leave the mitotic cycle indicates that the regulation of the activity of cAMP dependent protein kinase is essential in the maintenance of cellular regulation. Since the phenotype of bcy1 cells shows similarities to those of transformed mammalian cell lines (Table 1.2), it is not surprising that recent work has demonstrated that cAMP metabolism is closely involved in the mode of action of certain yeast oncogene homologues (Wheals, 1985).

Using antibody raised against p21, the 21 kDa protein product of the mammalian ras oncogenes, Papageorge, Defeo-Jones, Robinson, Temeles and Scolnick (1984) revealed ras related proteins in S. cerevisiae. DeFeo-Jones, Scolnick, Koller and Dhar (1983) probed the yeast genome using a DNA probe for mammalian ras genes and identified two sequences. The sequences, RAS1 and RAS2, showed extensive amino acid homology with

mammalian ras. The observations of Tatchell, Chafell, DeFeo-Jones and Scolnick (1984), that haploid yeast cells containing disruptions in both yeast RAS genes were unable to germinate, demonstrated that either of the two RAS genes was essential for spore germination. Their explanation was that the two RAS genes encoded products with similar or identical functions, a duplication of function also seen in several other essential genes (Rykowski, Wallis, Choe and Grunstein, 1981). The viability of spores containing disruptions at both RAS loci could be recovered by the expression of a plasmid based, human H-ras gene (Kataoka, Powers, Cameron, Fasano, Goldfarb, Broach and Wigler, 1985a). A reciprocal experiment has shown that a hybrid yeast/mammalian gene containing a portion of the activated yeast ras1 gene is capable of transforming mammalian NIH 3T3 cells (DeFeo-Jones, Tatchell, Robinson, Sigal, Vass, Lowy and Scolnick, 1985). The obvious functional homology between mammalian and yeast RAS gene products, suggests that there has been strong evolutionary pressure to conserve the pathways in which these essential proteins function. In the absence of conditional ras genes, Tatchell et al. (1984) were unable to attribute the ras1/ras2 phenotype to a specific effect on germination or a less specific effect on growth. This question was addressed by Kataoka et al. (1985a) who, by linking a ras complementing gene to the GAL10 promoter, were able to show that RAS genes are needed for continued growth because the cells arrested in the absence of galactose.

Investigation of the ras genes has proven them to resemble

genes involved in the metabolism of cAMP (Toda *et al.*, 1985). RAS2-val19, an allele containing a mutation analogous to that responsible for "activating" mammalian ras genes, leads to a phenotype typical of cells possessing the bcy1 mutation (Matsumoto *et al.*, 1982a). These cells are sporulation deficient, have reduced viability upon starvation, fail to accumulate reserve carbohydrate and have an elevated trehalase activity. The behaviour of cells containing the RAS1 and RAS2 disruptions is similar to that of cells lacking a functional adenylate cyclase. This similarity is extended by the observation that bcy1, isolated as a suppressor of the adenylate cyclase deficient cyr1 gene, is capable of suppressing the lethal phenotype of ras1/ras2 spores. Toda *et al.*, (1985) examined the effects of ras mutations on cAMP metabolism. They found that ras1 and ras2 mutations depressed cAMP levels but that RAS2-val19 elevated cAMP levels. In this way ras1/ras2 cells could mimic the effects of cyr1, and RAS2-val19 would have the same effect as IAC or bcy1 (Uno *et al.*, 1983a).

Casperson *et al.* (1983) demonstrated that yeast adenylate cyclase was regulated by GTP. Experiments on the adenylate cyclase of ras mutants proved ras1/ras2 cells to be insensitive to GTP stimulation. RAS2-val19 cells were also insensitive to GTP stimulation. However, their high adenylate cyclase activity would suggest that the RAS2-val19 disruption results in maximal stimulation of adenylate cyclase by a putative GTP binding protein (Casperson *et al.*, 1983), even in the absence of GTP. Given the similarity of phenotypes it is inevitable that the ras

genes would be compared with those genes affecting cAMP metabolism. Cautiously Toda et al. (1985) indicated that although they had shown ras genes to influence adenylate cyclase activity, the nature of the interaction between the two is not yet known. Evidence that ras proteins do not functionally interact with G protein complexes (protein complexes that regulate adenylate cyclase in mammalian cells), and that ras proteins are stimulated by GTP whilst G proteins are not (Broek, Samiy, Fasano, Fujiyama, Tamanoi, Northup and Wigler, 1985), suggests that the RAS proteins may not be functionally analogous to mammalian G proteins. Preliminary experiments have failed to show functional interaction between mammalian or yeast RAS proteins and mammalian adenylate cyclase (Broek et al., 1985). The finding that yeast adenylate cyclase, synthesised in Escherichia coli, is unresponsive to yeast RAS proteins, has several explanations (Kataoka, Broek and Wigler, 1985b). The favoured explanation, that yeast RAS proteins interact with adenylate cyclase via intermediates that are absent in E. coli, is consistent with those results that indicate functional differences between yeast RAS proteins and mammalian G proteins.

#### cAMP AND YEAST DEVELOPMENT

Cells of S. cerevisiae have several alternative fates after entering G1 and before completing start. It is during this phase of the cycle that the growth necessary for the attainment of a critical size is carried out (Johnston and Singer, 1983; Singer and Johnston, 1983). The mode of proliferation used by

S. cerevisiae results in the production of a bud that is smaller than the mother cell from which it was derived (Hartwell and Unger, 1977). Consequently the two individuals from a single division spend unequal times traversing G1. To ensure cellular viability, nutrient deprivation results in a non-growing G1 arrest (Johston et al., 1977). Mating pheromones arrest the cell cycle but allow the continued growth necessary for mating (Thorner, 1981). Although these responses are clearly different they all lead to the arrest of the cell cycle at a point prior to the completion of start. Thus we can describe the physiology of any cell that has not yet traversed start in one of three ways. A) Non-growing and non-shmooing because they are nutrient arrested (analogous to class II start mutations). B) Growing but non-shmooing (analogous to class Ib start mutations) because their inadequate volume restricts entry into mitosis. C) Growing and shmooing (analogous to class Ia start mutations) in response to mating pheromone. One could say that since these phenotypes are the cellular response to environmental assessment at start, a mutation that mimics any of them should be considered a genuine start mutant. This form of classification helps to explain the appearance of class Ib start mutants, a group that can not be accommodated in Reed's (1980) narrower definition of a start mutant; "Class-I start mutants should be equivalent to cells arrested by mating pheromone". cdc28 and cdc39 exemplify class Ia start mutants, cdc62 and cdc63 fall into class Ib (Bedard, Johnston and Singer, 1981), and cdc25, cdc35, cyr1 and cdc60 are considered to be class II. As a corollary it has proven possible

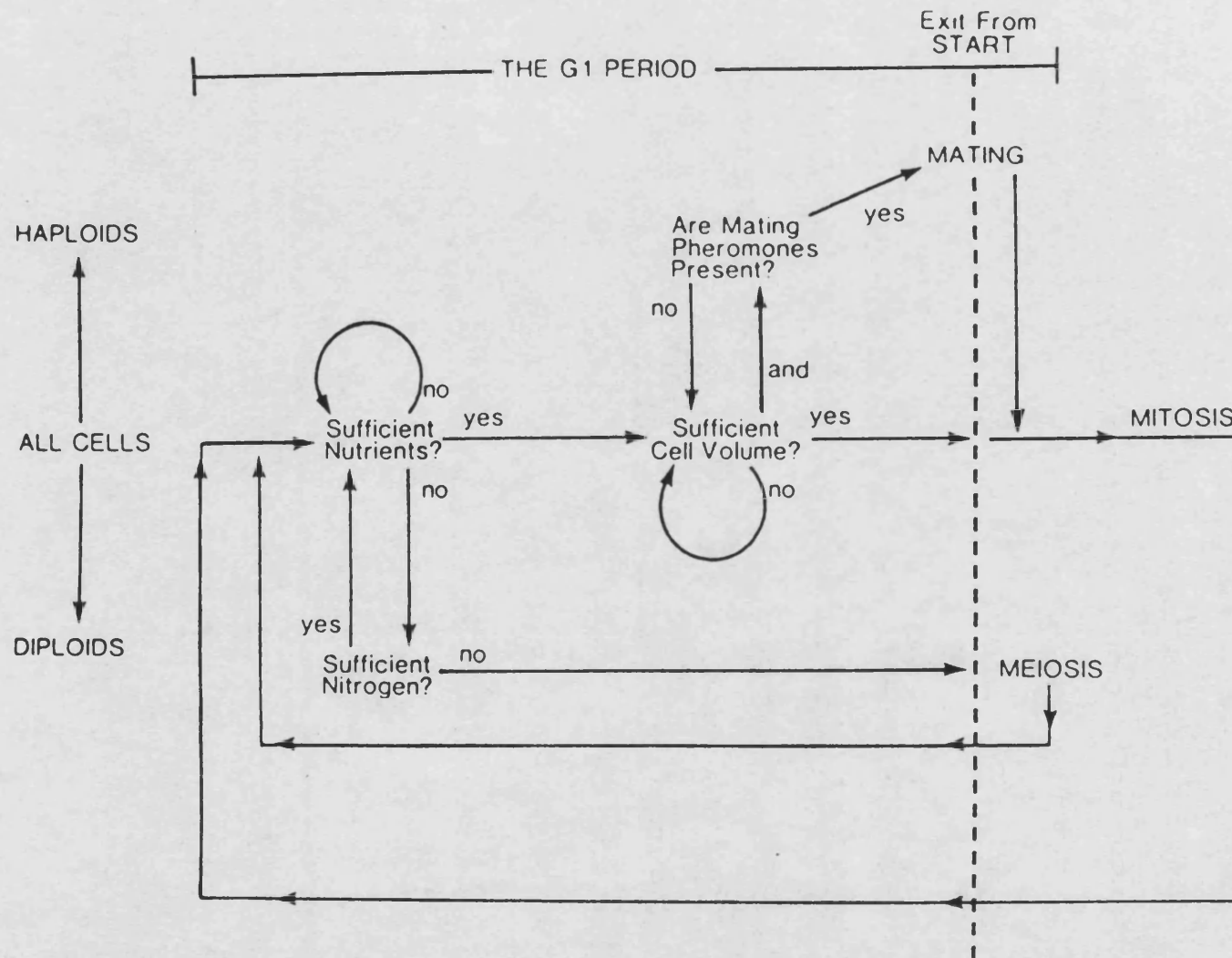
to isolate mutants that are effectively constitutive for these functions, failing to arrest at these block points because of inability to detect or respond to influential signals. Those failing to respond to nutrient starvation include whi2 (Carter and Sudbery, 1980; Saul, Walton, Sudbery and Carter, 1985), ard1 (Whiteway and Szostak, 1985), bcy1 (Matsumoto et al., 1982a) and RAS2-val19 (Toda et al., 1985); whi1 (Sudbery, Goodey and Carter, 1981) is a mutant initiating a cell cycle before growing sufficiently; and some of the ste (Hartwell, 1980) mutants are no longer responsive to mating pheromone. Although the effort has concentrated on the class I start mutants, the pleiotropic effects of the cyr1 mutation, acting epistatically on aspects of mating and sporulation illustrate the importance the cell places on regulation of start by nutrient availability. In many respects the response to nutrient supply could be regarded as the major input to start. In general growth is directly dependent upon the nutrient supply, and since both cell size and pheromone response are growth dependent they must ultimately be subject to regulation by nutrients.

An alternative way to view the start process is by using a flow diagram to illustrate the questions a cell "asks" prior to initiating its response at the start of the cell cycle (Figure 1.2). Although this diagram suggests that external influences are assayed in an ordered sequence during specific, finite regions of G1, it is more likely that assaying continues throughout the cell cycle but that the ability to respond is



Figure 1.2      The START Algorithm

This diagram depicts the rules a cell follows in the regulation of its development. Differences in the behaviour of haploid and diploid cells are illustrated by arrows above and below a central pathway of influences common to both. Following the arrows from left to right, any response that forces a digression from the central path ( which includes the pheromone question in the case of haploids) prevents traverse of the dashed line representing "exit from start". In this case start is taken in its widest sense to mean, the area of G1 over which cell cycle regulation occurs. Thus, exit from start is the point at which cells are committed to escape from G1. Consequently, cells failing to pass this point are considered to be G1 arrested. The nature and duration of this arrest is determined by the point of digression and the subsequent route taken to effect its release.



restricted to periods prior to the point we term "exit from start". It is clear that response to nutrient supply precedes all others and that a positive response leads to growth. However, the implied order following this is simply a constraint of graphical representation, and it is probable that subsequent "questions" would be concurrent and independent of each other. Start has been described as the point past which cells are committed to a mitotic cycle. This stringent description of start excludes the possibility of cells traversing G1 via pathways other than mitosis. Since sporulating cells execute the CDC4 step (the first event after the execution of the CDC28 step) they can be said to have traversed start on a pathway independent of mitosis. In the light of this a more flexible description of cells that have "started" would be those that have past the point at which environmental regulators are capable of inducing G1 arrest. Any cell not traversing start is in a G1 state but it should be emphasised that the biology of cells transiently traversing G1 is quite different to those undergoing G1 arrest induced by mating pheromones or nutrient depletion.

#### The high/go low/stop model

We propose an explicit model in which cAMP plays a major role in the integration of the various inputs to start. cAMP, acting through cAMP dependent protein kinase, would be responsible for the regulation of specific proteins in a cascade type system. The basis of the model is that effectors reducing the concentration of cAMP lead to G1 arrest. However, although a high level of cAMP is necessary to ensure traverse of start it is

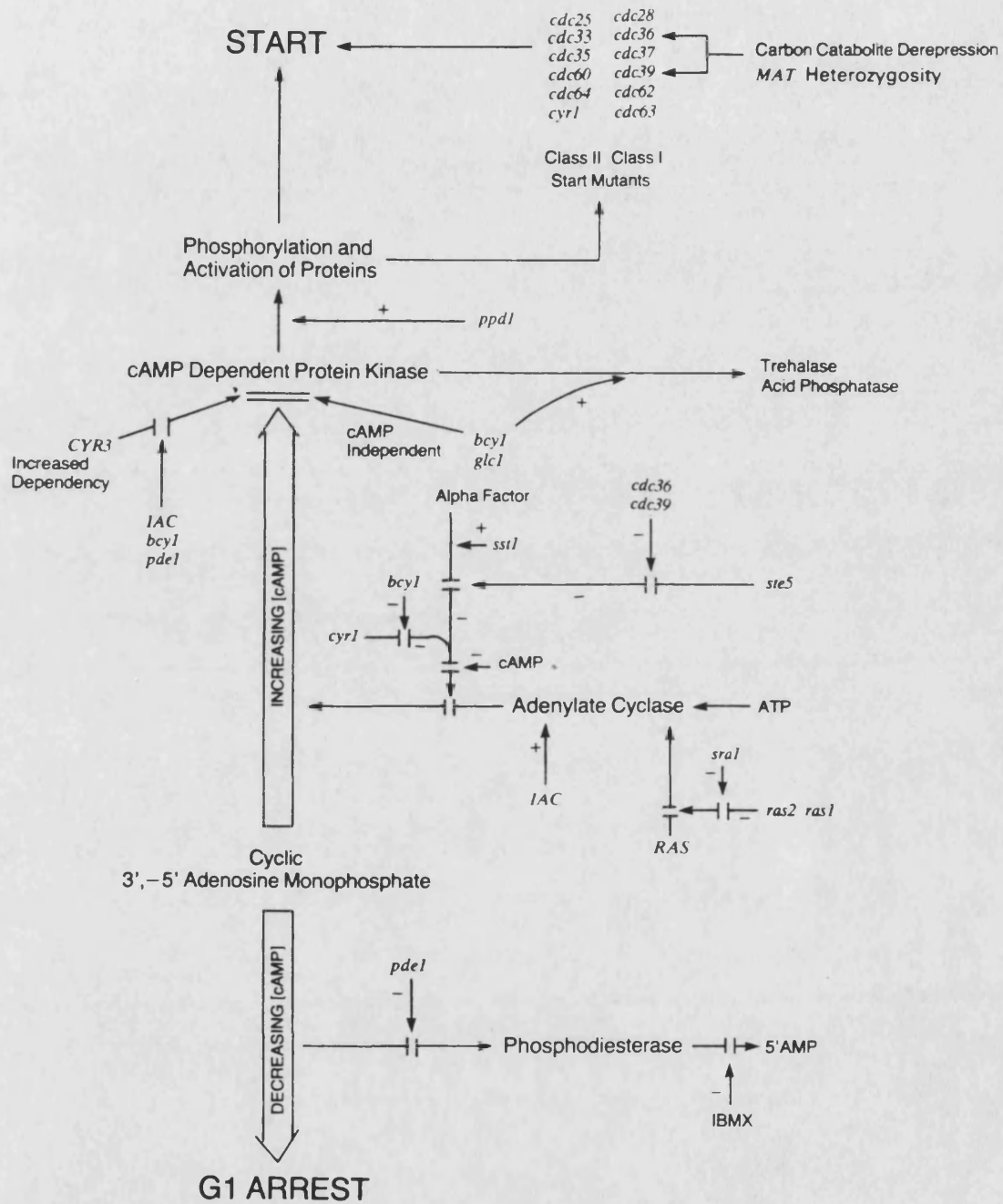
not in itself sufficient. Thus cAMP acts as a master switch capable of preventing start in the absence of favourable conditions. cAMP is a metabolite that has been implicated in the regulation of all pathways from start. Its ability to stimulate protein kinase accounts for the pleiotropic effects that such an "indicator" molecule must have. Figure 1.3 is a diagrammatic representation of the model. Centrally and on a vertical axis is placed cAMP and changes in its concentration. Decreased levels lead to G1 arrest and increased levels lead to traverse of start via the activation of cAMP dependent protein kinase and consequential protein phosphorylation. A number of the enzymes involved in cAMP metabolism (in its widest sense) are shown. On this framework we have also attempted to fit the data pertaining to many of the mutations affecting cell cycle regulation, the majority of which can be unambiguously assigned a position. The figure should serve as a useful reference since it indicates, where known, the nature of the mutations and may serve to allow predictions to be made concerning interactions between the different mutations.

The essence of the model is that the major (but not the only) determinant of cellular development is the in vivo level of cAMP. External and internal inputs mediate their effects via alterations to the level of cAMP. In turn the level of cAMP affects the degree of activation of cAMP dependent protein kinase(s), which in turn activates other protein kinases downstream. One of these steps is probably involved in the completion of the start events leading to initiation of the cell

FIGURE 1.3      High/go Low/stop Model

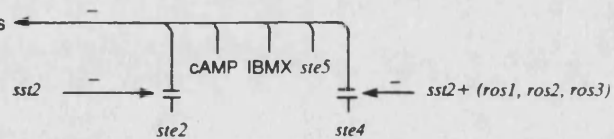
The diagram depicts factors affecting cAMP production and degradation. An increased level of cAMP leads to activation of cAMP dependent protein kinases and an enhanced level of phosphorylation of proteins, some of which are involved in subsequent traverse of start. Conversely, a decreased level of cAMP leads to G1 arrest. Mutations affecting cAMP metabolism or cell development are shown. Arrows marked with a positive sign indicate a stimulatory or synergistic effect; those marked with a negative sign have inhibitory or antagonistic action on arrows crossed with a double bar. A brief description of all of the mutants is given in Table 2. See the text for further explanation.

### The High/Go Low/Stop Model



Class II Start Mutants    Nutrient Starvation    ←    *bcy1*, *whi2*, *sra1*, *ard1*, *ppd1*

Class I Start Mutants      Mating Pheromones



cycle proper. The least well characterised stage is the link to the traverse of start.

The high/go, low/stop model is ultimately dependent, not upon the concentration of cAMP, but the activity of cAMP dependent protein kinase. There is evidence to suggest that the activity of this phosphorylating enzyme is affected by other metabolites. Uno, Matsumoto, Adachi and Ishikawa. (1984) indicate that high concentrations of cIMP are partially capable of supporting the growth of CYR3 mutants in the absence of cAMP, presumably by playing the role of a molecule capable of dissociating the regulatory and catalytic subunits of cAMP dependent protein kinase. Although both cIMP and cGMP are effective competitors of cAMP for its site on protein kinase (Takai, Yamamura and Nishizuka, 1974; Gilman, 1970; Hixson and Krebs, 1980), the activation constant for cIMP is two hundred times greater than for cAMP (Uno et al., 1982). These figures suggest that other cyclic nucleotides may act as effective inhibitors of cAMP dependent protein kinase by restricting cAMP access to sites on the regulatory subunit. In this scheme it is the effective concentration of cAMP, that is, its concentration relative to inhibitors rather than its absolute concentration, that is important. Regulation by two or more metabolites increases the resolution of the system, allowing the cell to inactivate cAMP dependent protein kinase by either decreasing the concentration of cAMP or increasing its competitive inhibitors.

The clear differences between the G1 arrest imposed by nutrient starvation and that imposed by mating pheromones cannot

be accommodated in a model in which a single compound has sole responsibility. Consequently, although cAMP may determine the phosphorylating ability of cAMP dependent protein kinase, it is the nature of the substrates available to the kinase that determine both the form of the arrest and the course taken upon its release. To date two enzymes have been identified as substrates for cAMP dependent protein kinase. Trehalase, the enzyme that catalyses the conversion of trehalose to glucose, is activated by phosphorylation (Ortiz et al., 1983; van Solingen and van der Plaat, 1975; Uno, Matsumoto, Adachi and Ishikawa, 1983b, van der Plaat and van Solingen, 1974). This explains the failure of mutants with an overactive protein kinase to accumulate carbohydrate. Acid phosphatase is the second proven substrate (Matsumoto et al., 1984); its ability to cleave phosphate groups makes it part of the phosphorylation/dephosphorylation system regulated by glucose. The recent isolation of a strain deficient in phosphoprotein phosphatase 1 (ppd1) (Matsumoto, Uno, Kato and Ishikawa, 1985b), has allowed an investigation of the relative importance of phosphorylation and dephosphorylation in the cell cycle. Since mutants with reduced phosphorylating ability (cyr1, cyr2 and CYR3) G1 arrest prematurely, while those with enhanced ability have deregulated proliferation (bcy1, IAC, pde1 and ppd1), Uno et al. (1983a) concluded that "phosphorylation of cellular proteins is indispensable for cell cycle, but dephosphorylation is not". Although this statement is correct if cell cycle is taken to mean "continued mitotic cycle" the characteristics of



enhanced phosphorylators clearly indicate that control over both phosphorylation and dephosphorylation is necessary for the regulation and integration of the component pathways that define the cell cycle.

### CONCLUSIONS

The connection between cAMP and the various developmental fates of S. cerevisiae cells has been established in some cases but remains obscure in others. The clearest evidence is for a direct role for cAMP in a nutrient response at the start event. Since both mitotic and meiotic development are dependent on the execution of start, cAMP has a direct role in both of these processes. There is clearly an indirect role for cAMP in mating since it is prevented by lack of growth, but a more direct effect (such as via mating pheromone on adenylate cyclase) remains to be confirmed. Similarly, although a correct starvation response is required for sporulation, the major role of cAMP may be in nitrogen metabolism regulation. Furthermore, although cAMP does have a role in catabolite repression/derepression, it is again indirect and not analogous to the system in prokaryotes. A substantial difficulty in much of the analysis reviewed is that it is difficult to disentangle indirect effects of cAMP via growth, from possible direct effects. The only clear evidence is for a role in growth via start. As yet, the nature of the informational inputs into the start process from mating and size control are unknown but cAMP is not a likely candidate since elevation of cAMP levels by any of the three inputs could lead to traverse of start. This would make each of the three inputs both necessary and sufficient whereas each alone is necessary and insufficient. Protein kinases could still be involved but activated by molecules other than cAMP.

If cAMP has a role in cell cycle regulation, then what

regulates the cAMP levels? Evidence from studies on the RAS mutants indicates that the activity of adenylate cyclase in the absence of GTP stimulation is sufficiently low to give a phenotype similar to mutants lacking a functional adenylate cyclase (cyr1). The RAS2-val19 mutation leads to enhanced levels of cAMP, possibly by maximal stimulation of adenylate cyclase via a putative GTP binding protein, and leads to a bcy1 like phenotype. Consequently, although the regulation of adenylate cyclase by other effectors cannot be discounted, it is clear that GTP regulation would, in their absence be sufficient to modulate adenylate cyclase activity over a range that would result in maximal and minimal levels of cAMP dependent protein kinase activity. The recent observation that conditions leading to sporulation cause a fall in intracellular GTP levels, provides in vivo evidence of the involvement of GTP in cellular development (Varma, Freese and Freese, 1985). It has led to the proposal that "GTP may act as a "signal" molecule by binding to a protein which controls the onset of meiosis". One could speculate that this binding protein is a RAS gene product acting as an intermediate in the regulation of cAMP through adenylate cyclase.

What are the substrates of the cAMP dependent protein kinase that are needed to traverse start? Although they have yet to be identified it is interesting to note that the CDC28 gene product has a structure making it both a substrate for a protein kinase and a protein kinase itself (Lorincz and Reed, 1983). Indeed, an anti-CDC28 antibody coprecipitates both the CDC28 gene product and a protein which has been phosphorylated (Reed, Hadwiger and

Lorincz, 1985). Furthermore, the gene products of both CDC4 (Peterson, Yochem, Byers, Nunn, Duesberg, Doolittle and Reed, 1984) and CDC7 (John Rosamond, pers. comm.), the next genes "downstream" of start in the functional sequence map, have structures consistent with them being protein kinases. The most cogent hypothesis to present is that all are part of a protein phosphorylation cascade system. The homology of the S. pombe CDC2 gene to CDC28 (Beach, Durkacz and Nurse, 1982) and the ability of mutant yeast RAS genes to induce morphogenic transformations in mammalian cells (DeFeo-Jones et al., 1985) suggests that the biochemical and genetical control of proliferation has been conserved during evolution.

The immediate research objectives with regard to the involvement of cAMP in yeast growth and development will probably include, i) identification of the substrates for cAMP dependent protein kinase, ii) an attempt to find all the intermediates linking cAMP and start, iii) establishing the validity of a phosphorylation cascade system as the early set of events in the cell cycle, iv) looking at the effects of other cyclic nucleotides, v) investigating the role of cAMP independent protein kinases as potential mediators of size (or mating pheromone) informational inputs, and vi) determining exactly how RAS genes both respond to nutrients and affect adenylate cyclase. There is every likelihood that the cAMP system will be the first cellular proliferation control process that is understood at the molecular level.

### SUMMARY

Although the cAMP metabolism mutants have complex phenotypes, the data concerning these mutants can be simplified as follows.

1) Does cAMP have a role in the cell cycle? The aberrant cell cycles of these mutants indicate that cAMP has some involvement in cell cycle regulation.

2) How does cAMP act as a regulatory molecule? The observation that cyr1/bcy1 double mutants grow and behave as bcy1 mutants indicates that all cAMP effects are mediated via the phosphorylating activity of cAMP dependent protein kinase (Matsumoto et al., 1982a).

3) The mutants of cAMP metabolism can be placed into two groups based on their phosphorylating potential. Enhanced phosphorylators (bcy1, pde1, ppd1 and IAC) fail to G1 arrest upon starvation, they do not accumulate carbohydrate, and are sporulation deficient. Reduced phosphorylators (cyr1, cyr2, and CYR3) sporulate in rich media and arrest as class II start mutants in the absence of exogenously supplied cAMP (Matsumoto et al., 1985a).

4) The enhance phosphorylators and reduced phosphorylators are phenotypically similar to the spo1 and spd1 mutants respectively (Vezinhet et al., 1979). These genes are thought to be involved in a metabolic function involving non-fermentable carbon sources.

A secondary effect of mutations in these genes is to alter nitrogen repression of sporulation.

5) Enhanced phosphorylators exhibit the phenotype expected of cells failing to respond to nutrient starvation. Reduced

phosphorylators behave as cells receiving a false "starvation signal".

Interpretation of the phenotypic characteristics of the cAMP metabolism mutants has prompted Matsumoto et al to imply that the molecule has a regulatory role in all the developmental pathways open to yeast. Although the pleiotropic properties of these mutants do impinge upon many aspects of cell regulation, it is possible, in summarising the data, to conclude that the primary role of cAMP is in signalling the nutritional status.

TABLE 1.2     MUTATIONS AFFECTING THE CELL CYCLE.

<u>alg1</u>	Glycosylation mutant; Class II G1 arrest. (Klebl, Huffaker and Tanner, 1984)
<u>ard1</u>	Arrest deficient; No G1 arrest; No sporulation; (Whiteway and Szostak, 1985)
<u>bcy1</u>	Results in a cAMP independent protein kinase, due to deficiency of the regulatory unit; No G1 arrest; No sporulation; No carbohydrate accumulation; Suppresses <u>cyr1</u> ; Elevated trehalase; (Matsumoto <u>et al.</u> , 1982a)
<u>cdc19</u>	Structural gene for pyruvate kinase; Class II start mutant; Allelic to <u>pyk1</u> ;
<u>cdc25</u>	Class II Start; Sporulates in rich media; (Shilo, Simchen and Shilo, 1978)
<u>cdc28</u>	Class I Start; Arrest at the point of Alpha-Factor; Continues to grow; Diploids are sporulation deficient; (Shilo <u>et al.</u> , 1978; Reid and Hartwell, 1977)
<u>cdc33</u>	Class II start; (Reid and Hartwell, 1977)
<u>cdc35</u>	Class II Start; Sporulates in rich media; Allelic to <u>cyr1</u> ; (Shilo, <u>et al.</u> , 1978)
<u>cdc36</u>	Class I Start; As <u>cdc28</u> ; Suppressed in carbon catabolite derepression conditions and by <u>MAT</u> heterozygosity; (Shuster, 1982b; Reed, 1980)

- cdc37 Class I Start; Similar to cdc28; (Reed, 1980)
- cdc39 Class I Start; As cdc36; (Reed, 1980)
- cdc60 Class II Start; (Bedard, Johnston and Singer, 1981)
- cdc62 Class I Start; non shmooing; (Bedard et al., 1981)
- cdc63 As cdc62; Allelic to prr1 (Hanic-Joyce, 1985)
- cdc64 As cdc60
- cyr1 Adenylate cyclase deficient; Sporulates in rich media; Arrests as nutrient starved Class II start in the absence of cAMP; Suppressed by bcy1; (Matsumoto et al., 1982a)
- cyr2 Altered catalytic subunit of cAMP dependent protein kinase; suppressed by ppd1; Sporulates in rich media; Arrests as nutrient starved Class II start in the absence of cAMP; (Uno et al., 1984)
- CYR3 Altered regulatory subunit of cAMP dependent protein kinase requiring elevated levels of cAMP for release; G1 arrest in the absence of cAMP; Suppressed by bcy1; (Uno et al., 1982)
- glc1 Deficient in glycogen & trehalose; Proposed cAMP dependent protein kinase; Likely allelism with ppd1; (Ortiz et al., 1983)



- IAC Increased adenylate cyclase; Suppresses CYR3;  
No G1 arrest; No sporulation; No carbohydrate  
accumulation. (Uno et al. 1983a)
- pde1 Reduced phosphodiesterase 1 activity;  
Suppresses CYR3; No G1 arrest; No  
sporulation; No carbohydrate accumulation.  
(Uno et al., 1983a)
- ppd1 Phosphoprotein phosphatase 1 deficient;  
Suppresses cyr2; No nutrient starved G1  
arrest; No sporulation; Likely allelism with  
glc1; (Matsumoto et al., 1985)
- ras1 Homologous to mammalian ras sequences;  
Adenylate cyclase insensitive to GTP; Lethal  
in ras2 background; reduced [cAMP]i.  
(Tatchell et al., 1984; Toda et al., 1985)
- ras2 Homologous to mammalian ras sequences;  
Adenylate cyclase insensitive to GTP; Reduced  
[cAMP]i; Sporulates in rich media; Lethality  
in ras1 background is suppressed by bcy1;  
Overproduces glycogen. (Tatchell et al.,  
1984; Toda et al., 1985)
- RAS2-val19 RAS2 gene with a valine at position 19; No  
G1 arrest; No sporulation; No carbohydrate  
accumulation ; High levels of adenylate  
cyclase activity; Insensitive to GTP;  
Elevated [cAMP]i; (Toda et al., 1985; Kataoka  
et al., 1985)

- ros1 Relaxation of sterility; Allelic to cdc39;  
Suppresses ste4 in the presence of sst2;  
(Jenness, D., A. Burkholder, and L. Hartwell.  
Abstr. Conf. Yeast Genetics and Molecular  
Biology. 1982. H15. p.140)
- ros2 As ros1; NOT allelic to cdc39;
- ros3 As ros2
- spd1 Sporulation derepressed; Sporulates in rich  
media; Arrests as nutrient starved Class II  
start (Vezinhet et al., 1979)
- spo1 No Sporulation; Suppresses spd1; No G1  
arrest; No sporulation; No carbohydrate  
accumulation (Calvert and Dawes, 1984, Dawes,  
1983)
- sra1 Suppressor of ras; No G1 arrest; No  
sporulation; No carbohydrate accumulation.  
(Tatchell, K., L. Robinson, J. Cannon, R.  
Schultz, M. Breitenbach, D. DeFeo-Jones, G.  
Temeles, I. Sigal, and E. M. Scolnick. Abstr.  
J. Cell. Biochem. Supp. 9C. 1985. 1422.  
p.114)
- sst1 Supersensitive to mating pheromone; MATa  
specific; (Chan and Otte, 1982a)
- sst2 Supersensitive to mating pheromone; Non MAT  
specific; Interacts with ste2; Suppresses  
ste4 in the presence of ros1, ros2 or ros3;  
(Chan and Otte, 1982a, see ros1)

- ste2 Mating defective; Mat specific; May be component of alpha factor receptor; (Hartwell, 1980; Shuster, 1982a, see ros1)
- ste4 Mating defective; Suppressed by sst2 in the presence of ros1, ros2 or ros3; suppression by cdc36 or cdc39 is MAT type dependent; (Hartwell, 1980; Shuster, 1982a; see ros1)
- ste5 Mating defective; suppressed by cdc36 and cdc39; Adenylate cyclase insensitive to alpha factor; (Shuster, 1982a; Liao and Thorner, 1980).
- ste8 Cells are phenotypically diploid; Expression of silent mating type loci; allelic to sir3; (Hartwell, 1980).
- ste9 Cells are phenotypically diploid; Expression of silent mating type loci; allelic to sir4; (Hartwell, 1980)
- tra3 Defective regulation of amino acid biosynthesis; Sporulation deficient at the restrictive temperature; G1 arrest at the restrictive temperature; Renamed gcd1 (Shilo et al., 1978; Wolfner et al., 1975)
- whi1 Relaxed regulation over size constraints at bud initiation; (Carter and Sudbery, 1980)
- whi2 Reduced sensitivity to glucose starvation; No G1 arrest; Reduced carbohydrate storage; (Sudbery et al., 1981)

CHAPTER 2

THE EFFECTS OF  
GLUCOSAMINE ON YEAST

### INTRODUCTION

One of the major effectors of the growth rate of Saccharomyces cerevisiae is substrate availability. Limiting the supply of nitrogen, carbon or other nutrients causes cell cycle arrest during G1, effectively reducing the growth rate to zero (Johnston et al., 1977). The cell is able to regulate its metabolism in response to nutrients in very subtle ways. The degree of oxidative metabolism relative to fermentative metabolism is determined by the nature of the available carbon source. Glucose, the preferred substrate, is capable of inducing catabolite repression (Polakis and Bartley, 1965), a physiologically and metabolically adaptive state in which carbon is rapidly dissimilated through fermentation. As a result, energy production is rapid allowing growth to proceed at maximal rates. To ensure an adequate energy supply, less energetic non-fermentable carbon sources are metabolised further through oxidative metabolism. The low energy yield of substrates such as pyruvate or ethanol and their demand for increased production of catabolic enzymes leads to reduced cellular growth rates. The relationship between substrate, growth rate, and metabolism is generally fixed and can be summarised as, glucose= fast growth= catabolite repressed, non-fermentable substrate= slow growth= catabolite derepressed. Consequently, cells fermenting glucose differ from those oxidising pyruvate in both growth rate and degree of catabolite repression. The tight linkage of these two features complicates the differentiation of those observations

related to repression and those dependent on growth rate (Shuster, 1982b).

Resolution of this problem could be achieved if changes in growth rate were uncoupled from the biochemical and morphological changes that characterise repression/derepression. Since it would be impossible for cells metabolising pyruvate to attain the high growth rate of those using glucose, the favoured approach is to reduce their growth rate on glucose whilst maintaining their repressed state. Several methods of modulating growth rate have been tried, each has disadvantages that deny their use in this situation. Diffusion capsules are capsules containing substrates that allow their contents to diffuse into the medium at a constant rate along a concentration gradient (Pirt, 1971). Their disadvantage is that in batch culture the concentration of substrate would change rapidly in response to exponential population growth and increased consumption. Since it is the limited substrate supply that sets the growth, it would be difficult to establish a system in which a constant growth rate could be maintained over several generations.

A second option is the glucose limiting chemostat. Although technically complex this system has been used to great effect by Beck and von Meyenburg (1968). They have examined several aspects of growth and metabolism in response to glucose limitation and their results demonstrate that at growth rates characteristic of non-fermentable carbon sources, cells metabolising glucose have the enzyme pattern of derepressed cells. They indicate their belief that glucose "flux" is

involved in establishing the degree of repression. The conclusion is that although limiting the glucose supply is capable of modulating population growth, the reduced flux switches cells from a repressed to derepressed state. Consequently, this system cannot be used to obtain slow-growing repressed cells.

As a growth rate modulator Shuster (1982b) added a protein inhibitor to cells growing on glucose. Although this addition successfully reduced the growth rate, cells treated with non-specific protein inhibitors cannot be considered suitable controls for cells free of this treatment, since the action of protein inhibitors has not been fully characterised.

The shortcoming in methods dependent upon limiting glucose, is that the reduction in the flux of carbon catabolite repressors leads to a state of derepression. If this flux could be maintained even though the uptake of glucose is restricted, it may be possible to produce slow-growing repressed cells.

A number of glucose analogues have been examined for their effects on carbohydrate metabolism (Woodward, Cramer and Hudson, 1953). Those analogues found to be most effective had substitutions on the number 2 carbon atom. 2-deoxyglucose, was effective at inhibiting both aerobic and anaerobic fermentation and growth. D-glucosamine was less potent. It had no effect on aerobic fermentation and was less successful at suppressing growth on glucose. D-glucosamine has been examined in detail by several groups. Woodward and Hudson (1953) demonstrated that glucosamine could reduce the anaerobic fermentation of glucose in the manner of a competitive inhibitor. Burger and Hejmová

(1961), found that the accumulation of glucosamine could be inhibited by glucose. The structural similarity between glucose and glucosamine, and the observation that glucosamine would prevent growth on glycerol or ethanol (Furst and Michels, 1977) led to its examination as an inducer of catabolite repression (Hockney and Freeman, 1980). Furst and Michels (1977) demonstrated that glucosamine was capable of producing a repressed state similar to glucose repression. So effective was its ability to repress that several groups have used resistance to either glucosamine or 2-deoxyglucose in the selection of carbon catabolite repression resistant mutants (Zimmermann and Scheel, 1977; Michels and Romanmski, 1980; Lobo and Maitra, 1977a; Bailey and Woodward, 1984).

The observation that glucosamine was not metabolised at an appreciable rate (Burger and Hejmova, 1961) and therefore could not be used to support growth, led to the possibility that a glucose glucosamine competition would reduce growth rate without a significant change in the flux of catabolite repression inducers. It was found that this competition could modulate growth rate in a predictable manner and effectively uncouple growth rate and the degree of repression. Examining the nature of this repression revealed that glucosamine could inhibit the accumulation of glucose derived label by intact yeast cells and would act as a competitive inhibitor of hexokinase for glucose.



## RESULTS

Since D-glucosamine had been demonstrated to be an effective inducer of catabolite repression, it was hoped that a derepressive carbon source could be found that would support the growth of yeast in the presence of non-metabolisable glucosamine.

Figure 2.1 presents the results of growth experiments to examine this point. Clearly neither galactose nor glycerol can support significant growth in the presence of 1% glucosamine. Similar results were obtained with raffinose, cellobiose, mannitol, sorbitol, succinic acid, sodium acetate, ethanol, lactic acid and sucrose all added at 2%. A possible explanation is that glucosamine's structural similarity to glucose is sufficient to repress the production of enzymes necessary to metabolise these substrates. Suppression of growth on a wide range of substrates strengthens the claim that glucosamine repression is identical to that of glucose rather than a subset of it. The concentration of glucosamine effective at inhibiting growth on 2% sodium pyruvate, is indicated in Figure 2.2. Although concentrations above 0.1% were capable of preventing growth on pyruvate they could only reduce growth on 0.05% glucose. It is this observation of suppression that led to the establishment of a growth rate modulating system based on varying the glucose:glucosamine ratio.

The relationship between glucose and glucosamine with respect to growth rate, was quantified by a series of experiments in which cells were grown in YEP supplemented with various concentrations of glucose and glucosamine. The results of this are presented in Figure 2.3. It was found that for a given

FIGURE 2.1      INHIBITORY EFFECTS OF D-GLUCOSAMINE

The effect of D-glucosamine on the growth of S. cerevisiae D273-11a using glycerol or galactose carbon sources.

2% galactose with ( ■ ) and without ( □ ) 1% glucosamine.

2% glycerol with ( ● ) and without ( ○ ) 1% glucosamine. At time zero cells were inoculated at the same initial density from a pyruvate grown starter culture.

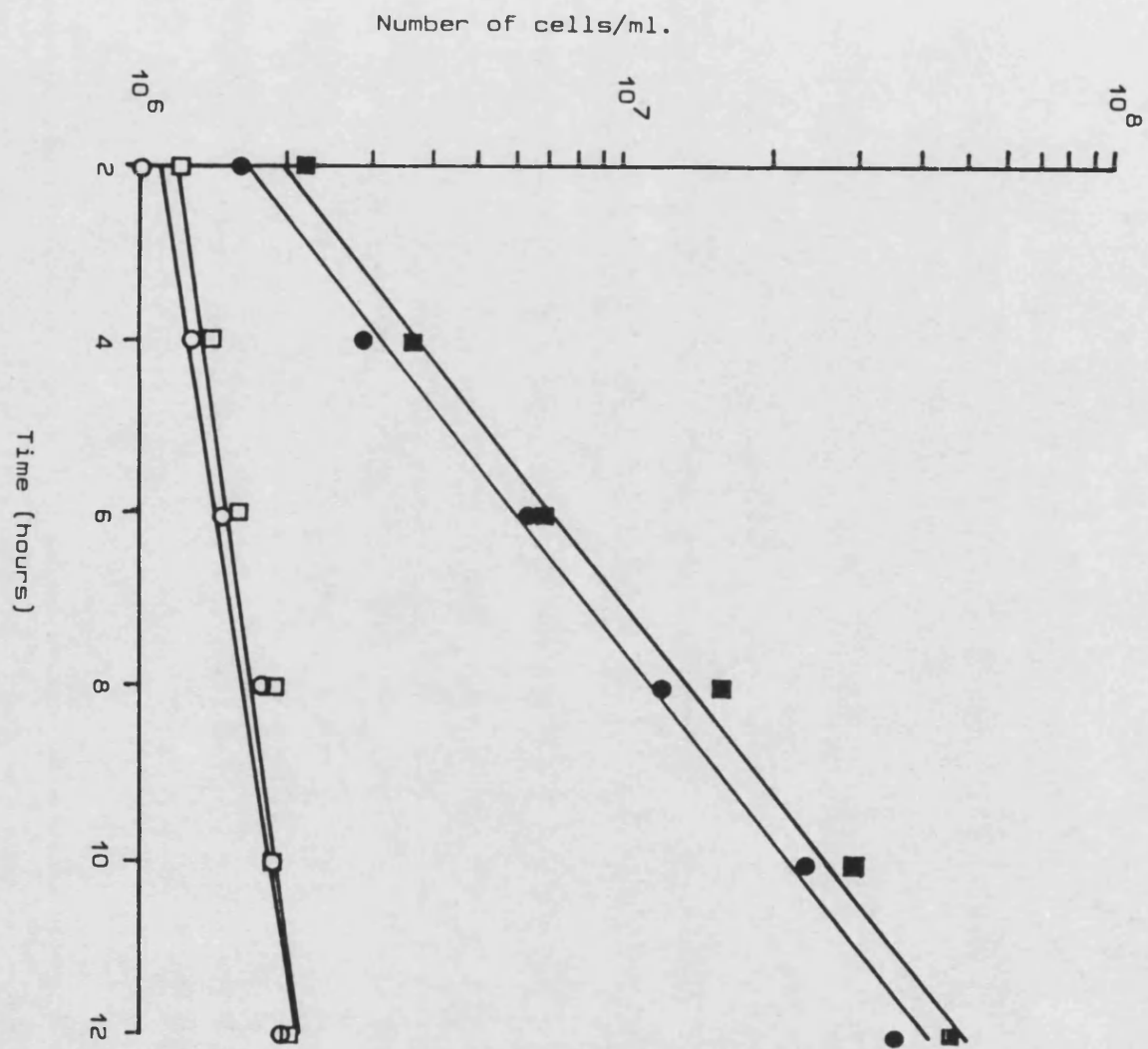


FIGURE 2.2      MINIMUM INHIBITORY CONCENTRATION OF GLUCOSAMINE

The effect of increasing concentrations of glucosamine on the ability of S. cerevisiae 2-15 to grow on 0.05% glucose ( □ ) or 2% sodium pyruvate ( ○ ). Cells were initially inoculated to the same starting density from a glucose grown starter culture. The final cell density was normalised with respect to the initial cell density. The line was fitted using a regression analysis to determine the "best fit."

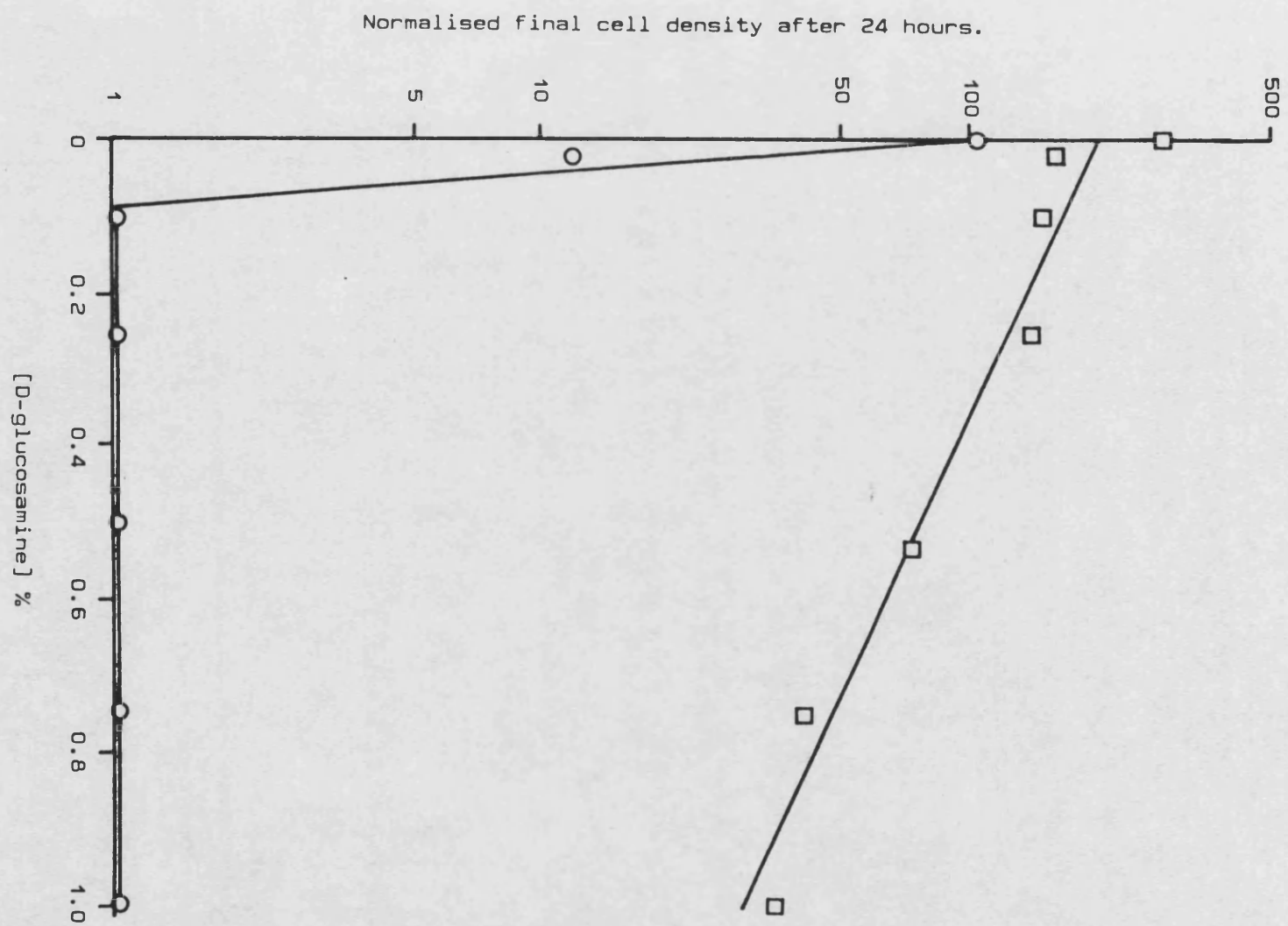


FIGURE 2.3 GLUCOSE:GLUCOSAMINE RATIOS

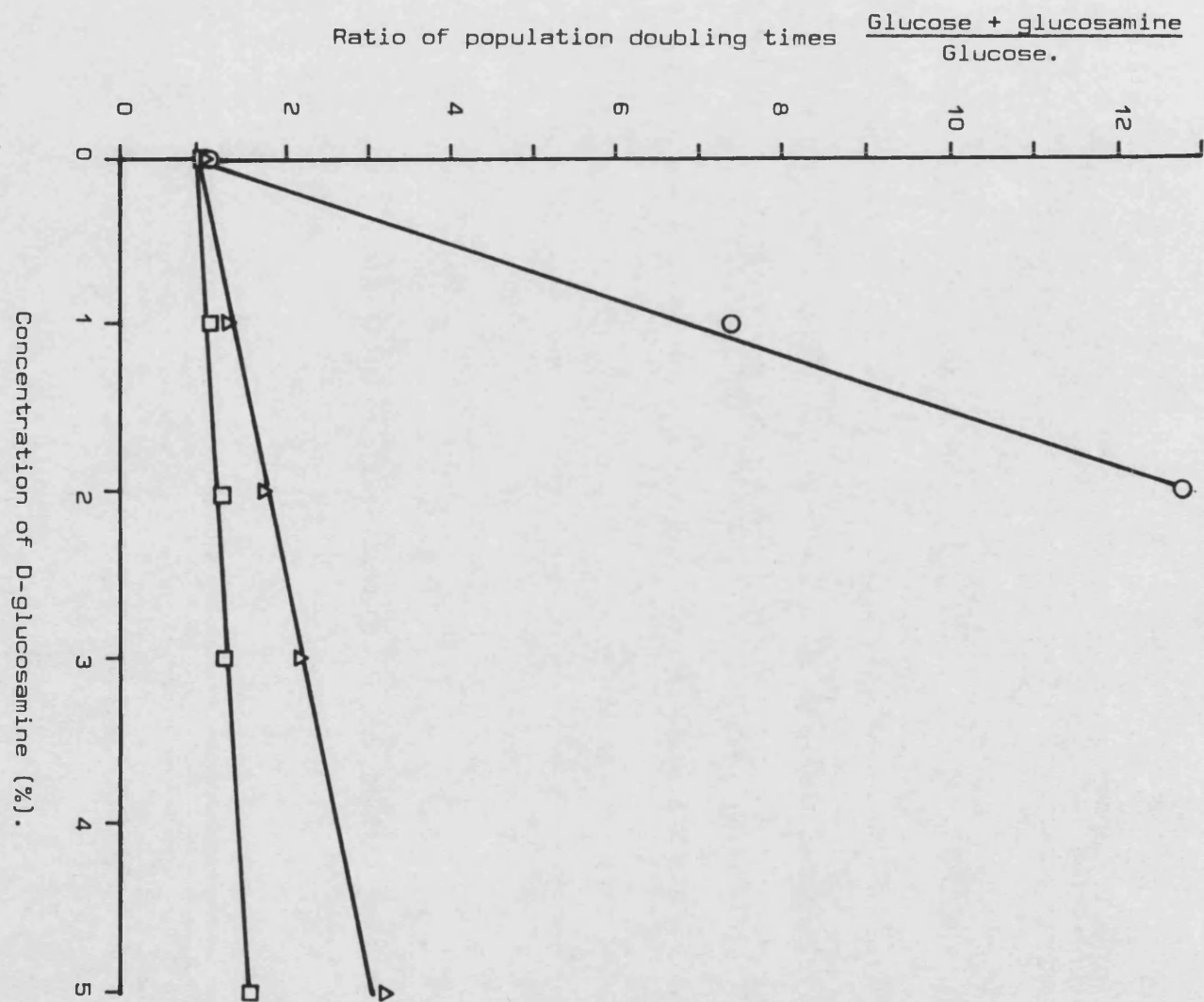
The effect of varying the glucose:glucosamine ratio on the growth rate of S. cerevisiae SR665-1. Cells were grown at 23°C on a YEP base supplemented with various concentrations of glucose and glucosamine.

( O ) 0.5% glucose.

( Δ ) 1.0% glucose.

( □ ) 2.0% glucose.

Lines were fitted as in Figure 2.2



concentration of glucose the glucose:glucosamine ratio would affect the growth rate in a predictable, linear manner. Although this ratio is important the absolute concentration of glucose also has a profound effect upon growth. This indicates that if the reduction in growth rate were due to the competition between glucose and glucosamine limiting the supply of glucose for metabolism, the system has a greater affinity for glucose than glucosamine. If the two substrates were of equal affinity the growth rates on 0.5% glucose:1% glucosamine, 1% glucose:2% glucosamine, 2% glucose:4% glucosamine, would be the same.

An understanding of the relationship between the glucose:glucosamine ratio and the growth rate allows the manipulation of growth rate by the addition of appropriate concentrations of glucosamine to cells metabolising glucose. This system has been used to produce exponentially growing populations of cells whose growth rate on glucose is below that of cells using 2% sodium pyruvate (Figure 2.4). The ability of glucosamine to successfully compete with glucose suggests that its uptake, although less efficient than that of glucose, occurs at a significant rate. Since the rate at which it is metabolised is very slow, the intracellular concentration should reach equilibrium rapidly at which point any toxicity might be expected to exert effects on viability. Consequently, the continued exponential growth of cells on 2% glucose + 1.4% glucosamine indicates that glucosamine has no toxic effects on the cell.

Invertase (EC 3.2.1.26) catalyses the hydrolysis of sucrose



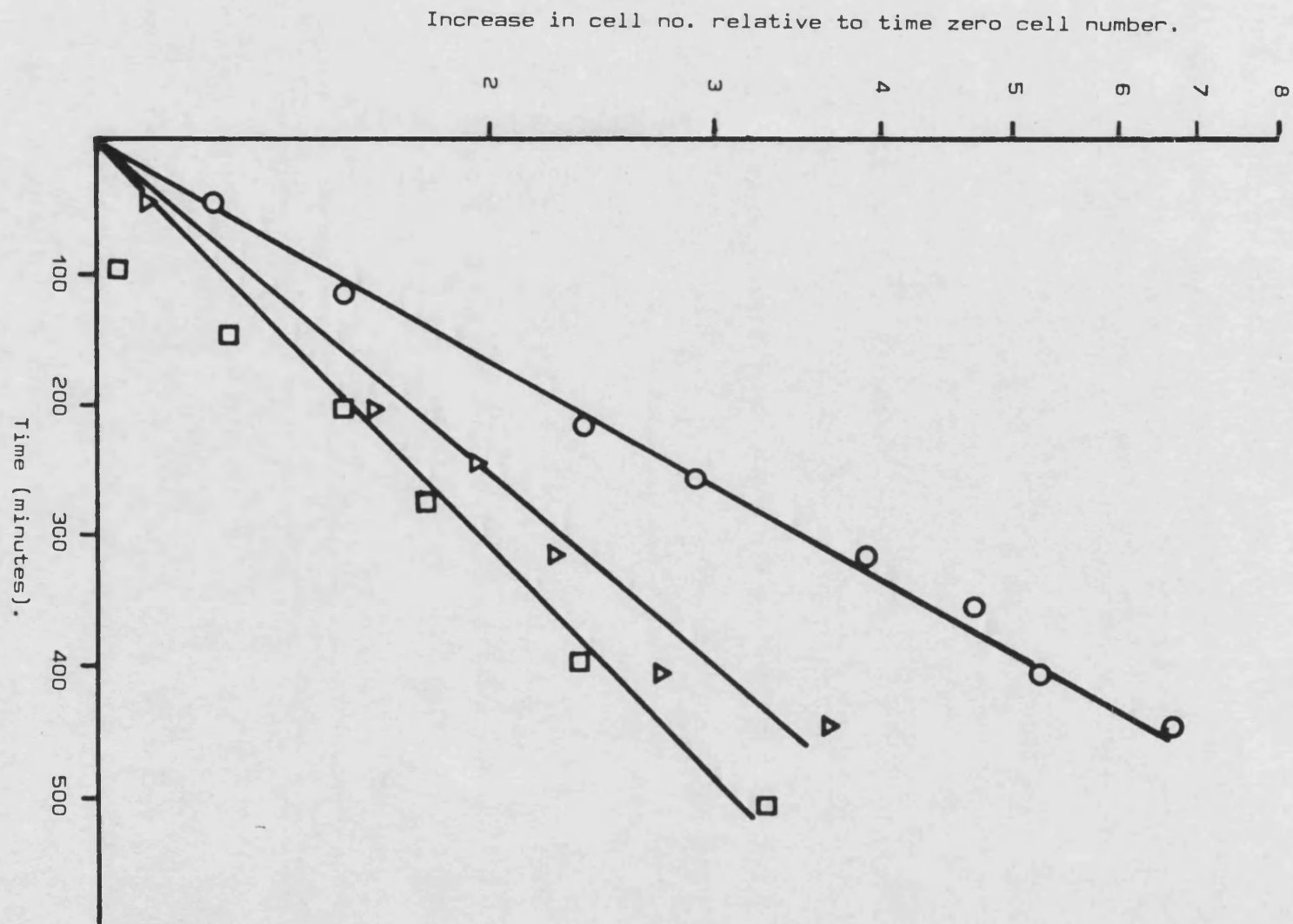
FIGURE 2.4      GROWTH RATES

Population growth of S. cerevisiae<sup>SR665-1</sup> growing at 23°C in YEP batch culture with various carbon sources. Cultures were inoculated at time zero.

( O )    2% glucose.

( Δ )    2% sodium pyruvate.

( □ )    2% glucose + 1.4% glucosamine.



to fructose and glucose, and is subject to glucose regulation of its synthesis (Dodyk and Rothstein, 1984). It has frequently been used as an indicator of the cells repression status (Bailey and Woodward, 1984; Matsumoto et al., 1983c). Figure 2.5 depicts the relationship between carbon source, growth rate and invertase activity. Although the addition of 1% glucosamine has a dramatic effect on the growth rate, it has little effect on invertase activity. Relative to the pyruvate derepression control, both the fast growing glucose, and slow growing glucose + glucosamine populations have repressed invertase activity. This indicates that the addition of glucosamine can be used to manipulate growth rate independent of effects on catabolite repression.

To investigate further the putative glucose:glucosamine competition suggested by Figure 2.3, glucose accumulation and its inhibition by glucosamine was studied. Figure 2.6 describes the kinetics of label accumulation. This plot (Lineweaver and Burk, 1934) extrapolates experimental data giving a  $V_{max}$  of  $21\text{nM}/10^8$  cells/ml/min. Half the maximum rate of accumulation ( $K_m$ ) is achieved at 1mM glucose. The effect of glucosamine on the rate of accumulation of label by intact yeast cells was examined (Figure 2.7). The observations of this work are that (i) the rate of accumulation of label increases in line with the external glucose concentration and (ii) increasing concentrations of glucosamine have increasingly inhibitory effects on accumulation.

Although it is clear that glucosamine acts as an inhibitor of label accumulation and therefore glucose uptake, the experimental data do not fit the standard kinetic plots of a competitive

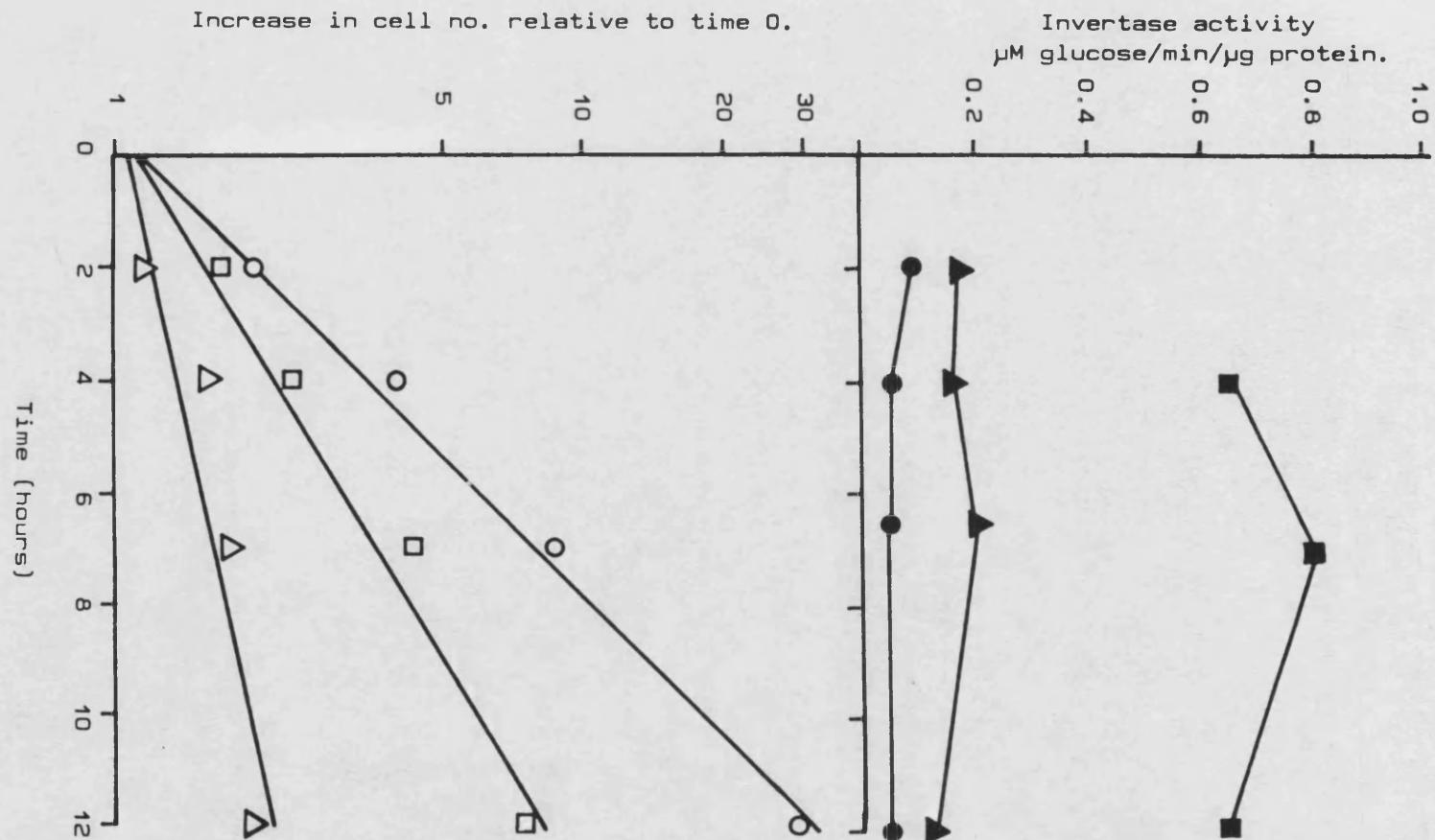
**FIGURE 2.5**      INVERTASE ACTIVITIES

The relationship between invertase activity (black symbols) and growth (open symbols) of S. cerevisiae SR665-1 cells using YEP+ various carbon sources. Growth curves fitted as in Figure 2.2.

(●○) 2% glucose

(■□) 2% pyruvate

(▲△) 2% glucose + 1% glucosamine.



**FIGURE 2.6      KINETICS OF LABEL ACCUMULATION**

Lineweaver-Burk plot to allow the determination of apparant  $V_{max}$  and  $K_m$  values for the accumulation of intracellular label derived from labelled glucose. The rate of accumulation ( $V$ ) is expressed in nanomoles glucose/ $10^8$  cells per minute. The line was plotted as in Figure 2.2.

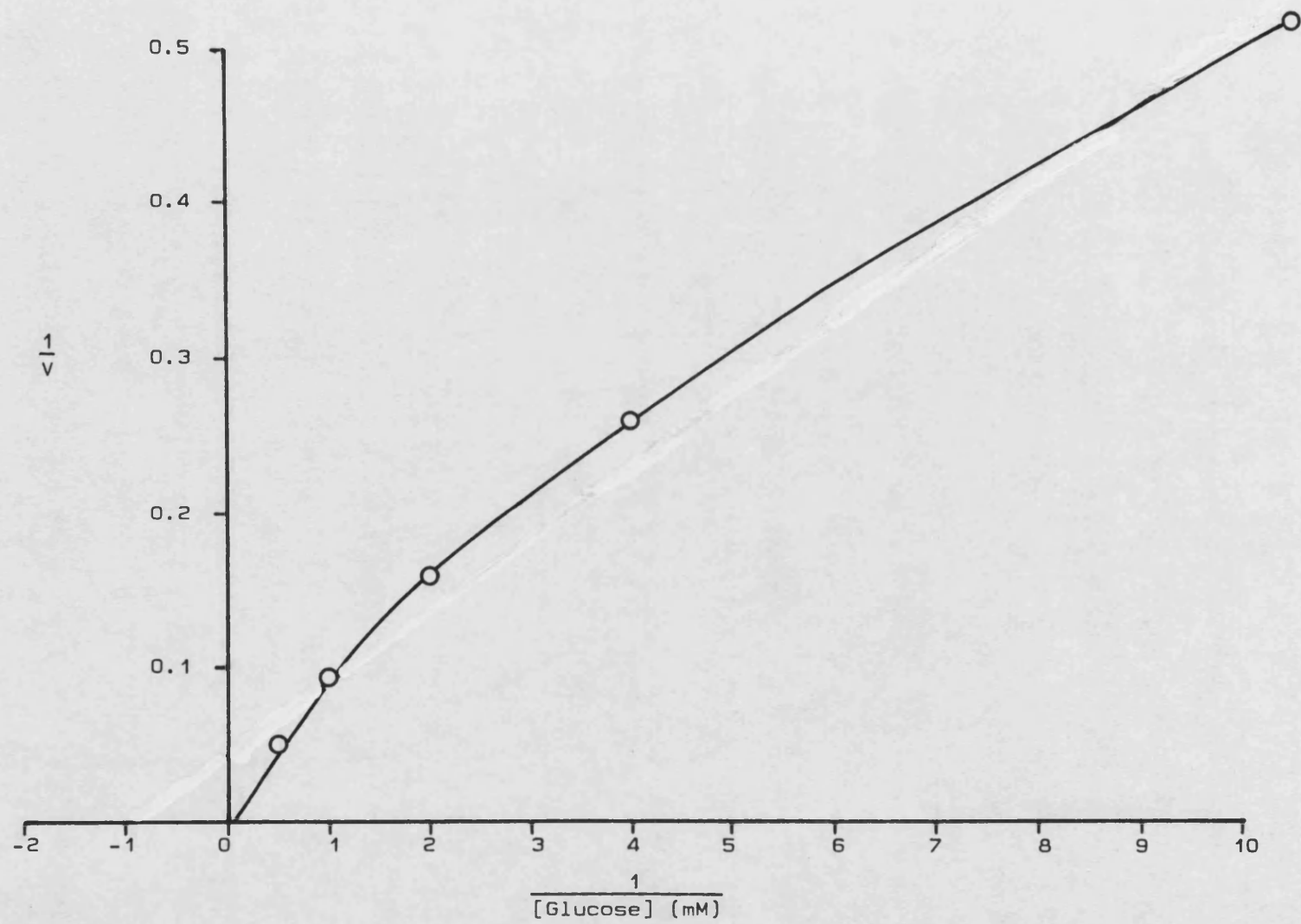


FIGURE 2.7      INHIBITION OF LABEL ACCUMULATION BY GLUCOSAMINE

The effect of glucosamine on the accumulation of labelled glucose by intact SR665-1 cells. The incubation solution contained various concentrations of labelled glucose and glucosamine, the accumulation was deemed to begin upon the addition of cells. Inhibition is expressed relative to the maximum observed accumulation which is assigned a value of 100%.

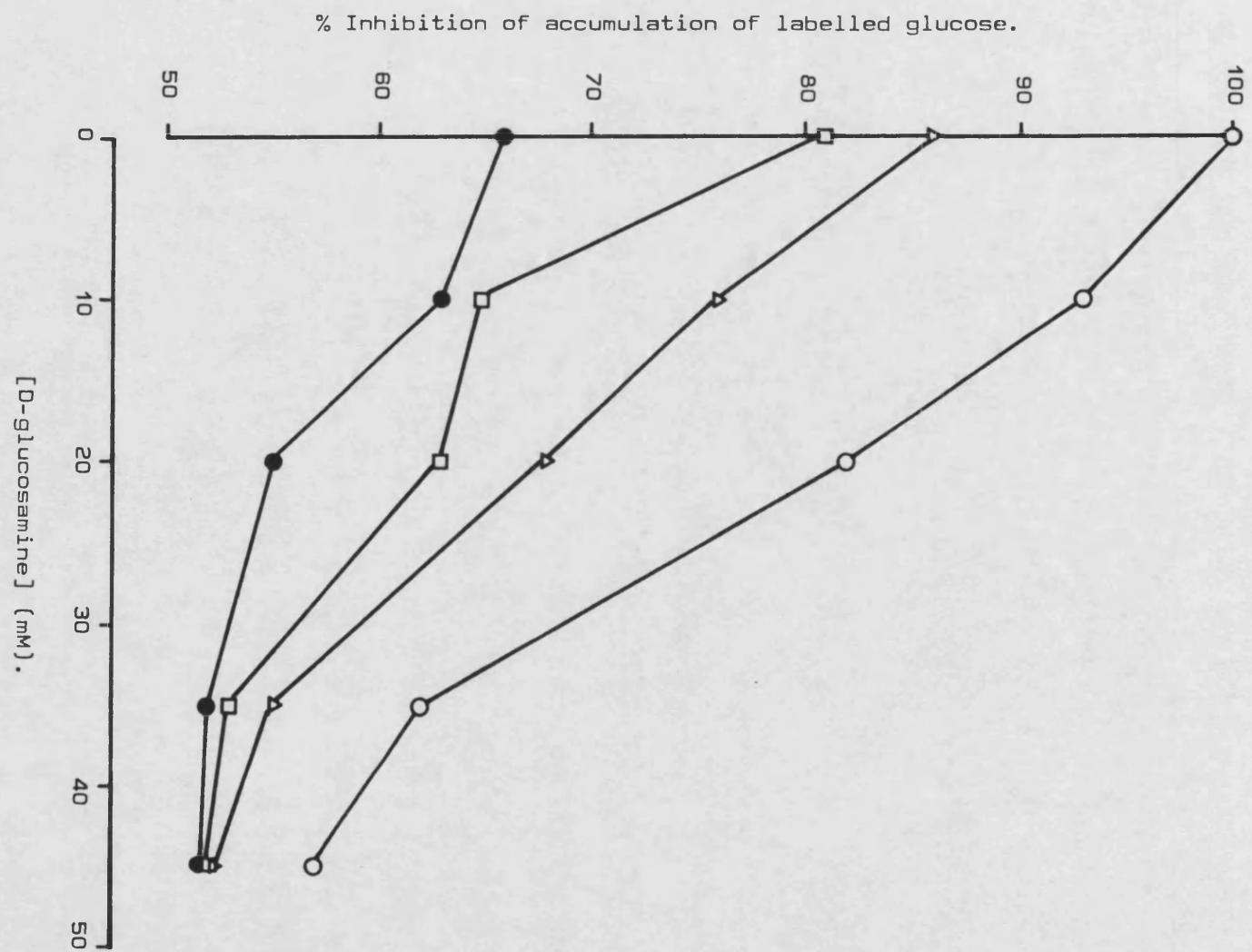
( ○ )    17.5mM glucose

( △ )    12.5mM glucose

( □ )    10.0mM glucose

( ● )    7.5mM glucose





inhibitor. The reasons for this are unclear however, since glucose uptake is probably a complex multi event system it is possible that glucosamine has differential effects on the various elements of the system.

Hexokinase (EC 2.7.1.1) phosphorylates glucose as the first step in its metabolism. Since facilitative diffusion is proposed to be involved in glucose uptake, hexokinase activity plays an essential role in uptake by maintaining the necessary concentration gradient. As an element of uptake hexokinase can be examined independently of membrane transport by using partially purified cell free extracts. A Lineweaver-Burk plot of hexokinase activity relative to glucose concentration (Figure 2.8) gives  $V_{max}$  and  $K_m$  values of hexokinase for glucose as 4.4mM and 0.19mM respectively. The ability of glucosamine to inhibit hexokinase activity was assayed at glucose concentrations around the  $K_m$  value. In this case the results generated fit the Dixon plot characteristic of competitive inhibitors (Dixon, 1953) (Figure 2.9). This plot gave a  $K_i$  value for glucosamines effect on the ability of hexokinase to phosphorylate glucose of 1.7mM.

FIGURE 2.8 THE  $V_{max}$  AND  $K_m$  VALUES OF HEXOKINASE FOR GLUCOSE

Lineweaver-Burk plot to allow the determination of the  $V_{max}$  and  $K_m$  values of hexokinase for glucose from partially purified cell free extracts of S. cerevisiae SR665-1. Hexokinase activity ( $V$ ) is expressed in micromoles NADPH formed per minute/mg protein. The line was plotted as in Figure 2.2.

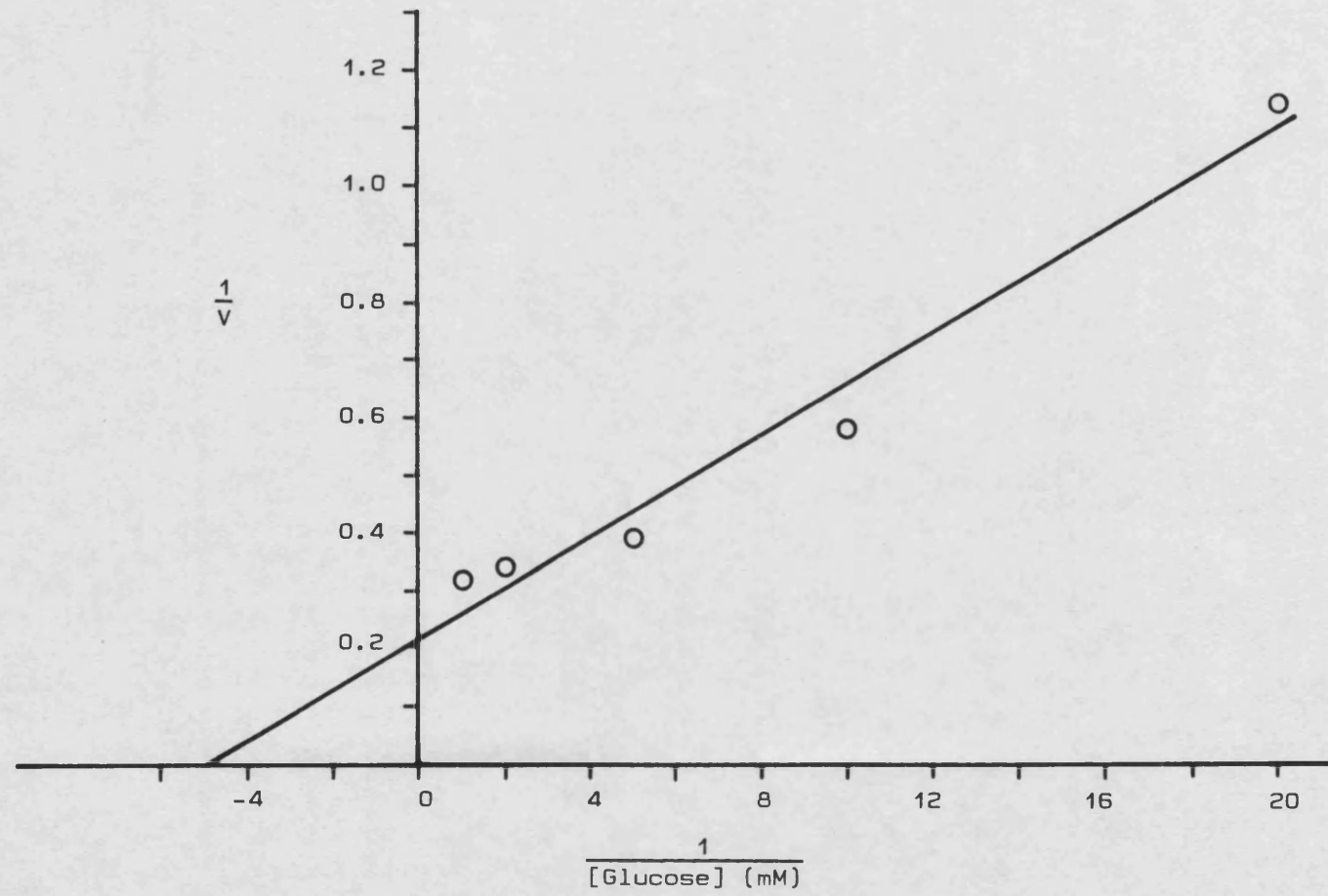


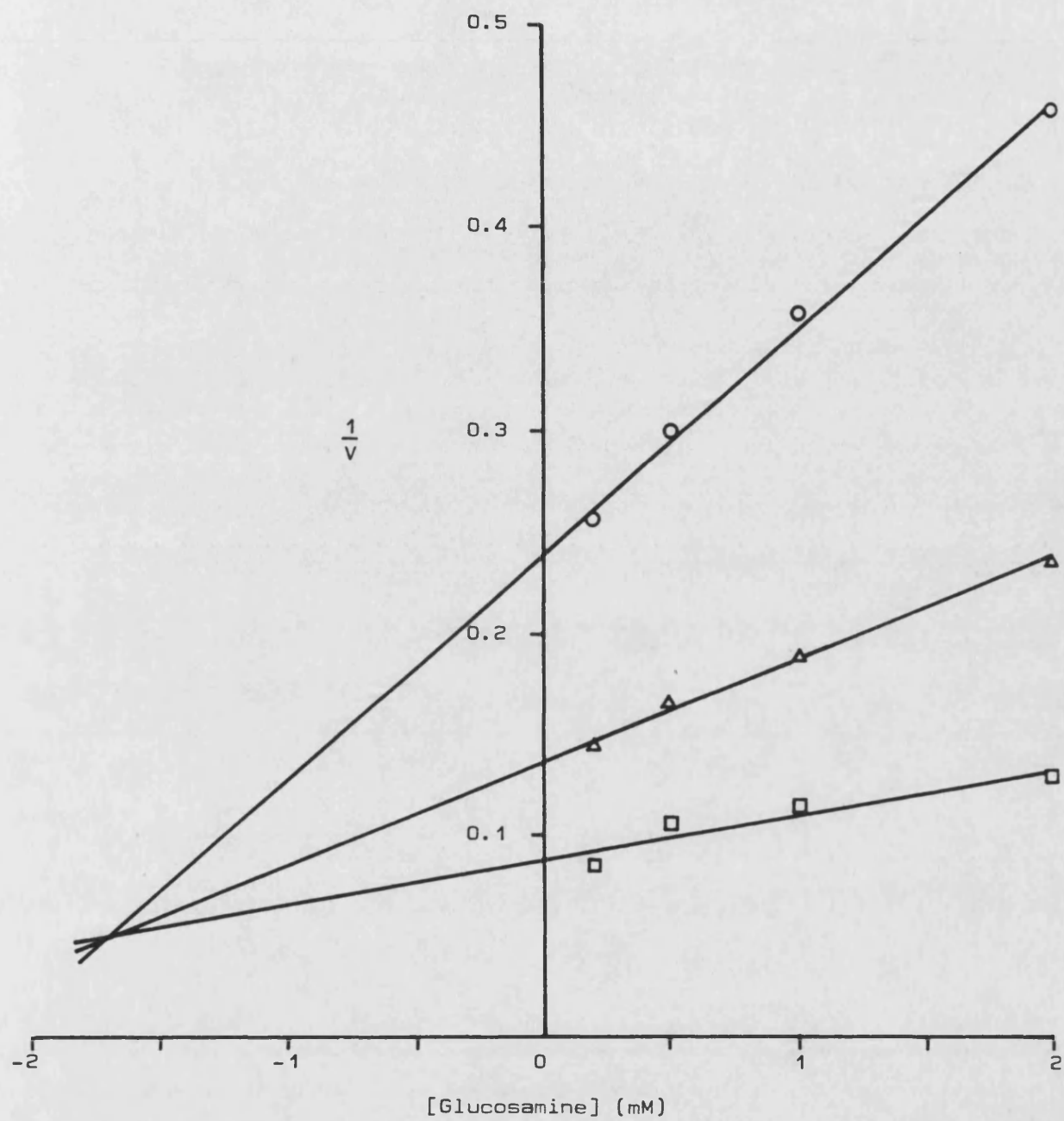
FIGURE 2.9      INHIBITION OF HEXOKINASE BY D-GLUCOSAMINE

Dixon plot allowing the determination of the  $K_i$  value of D-glucosamine for hexokinase. Hexokinase was assayed in partially purified cell free extracts of S. cerevisiae SR665-1 in the presence of various concentrations of glucose and glucosamine. Hexokinase activity (V) is expressed in micromoles NADPH formed per minute/mg protein.

( ○ ) 0.05mM glucose

( △ ) 0.10mM glucose

( □ ) 0.20mM glucose



### DISCUSSION

Carbon catabolite repression has been extensively studied, but as yet, its molecular trigger and the subsequent sequence of events involved in the establishment of its pleiotropic nature remain unknown. As a consequence of the pleiotropy it is difficult to differentiate primary and secondary features. The development of a system that uncouples growth rate and catabolite repression, allows a confident statement that responsibility for a change occurring as a result of a shift from repressive to derepressive conditions, is a consequence of a change in either the repression status or growth rate.

Although the definitive trigger of repression/derepression is unknown, the evidence that glucosamine and other non-metabolisable glucose analogues are effective inducers, eliminates the possibility that the "flux" of middle or late glycolytic intermediates are involved. This has been confirmed by Entian and Frohlich (1984) who compared metabolite concentrations in repression resistant mutants with those of the wild type and were unable to find significant differences. Nevertheless, the data are not inconsistent with the suggestion that a flux related to early glycolysis is the determining factor in repression. Recent evidence indicates that hexokinase plays a key role in establishing repression (Entian et al., 1984; Entian and Frohlich, 1984, and Entian and Mecke, 1982). The isolation of appropriate mutants has demonstrated that hexokinase isoenzyme PII contains separate domains with catalytic and regulatory functions. It is proposed that binding of a substrate to the

catalytic site produces a change in the regulatory domain triggering catabolite repression. This hypothesis explains why 2-deoxyglucose and glucosamine are capable of acting as inducers.

Since glucosamine is a substrate for hexokinase, it forms part of the "flux" that induces catabolite repression through hexokinase. Although glucosamine is a substrate for hexokinase its catabolism proceeds no further. Consequently, each glucosamine molecule bound provides the same trigger for repression as glucose, without its energetic contribution. The overall effect of this is to maintain the repression trigger but reduce the energy available from glycolysis with a consequential reduction in growth rate.

Glucose uptake is a contentious subject, the relationship between its transport across the membrane and its phosphorylation by hexokinase is not yet clear. Although there is agreement that movement across the membrane is by means of facilitative diffusion, the suggestion that hexokinase is involved in this process producing a "transport related phosphorylation" mechanism is disputed by some (Kuo and Cirillo, 1970). Nevertheless, the bulk of the evidence favours the active participation of kinases in glucose transport. Using hexose kinase mutants Bisson and Fraenkel (1983a) have demonstrated that yeast exhibits both high and low affinity uptake mechanisms for glucose. They report that where the wild type yeast had an uptake system with a  $K_m$  of approximately 1mM, a mutant strain lacking the three glucose phosphorylating enzymes hexokinase PI (h<sub>xk</sub>1), hexokinase PII (h<sub>xk</sub>2), and glucokinase (glk) showed a system with an affinity of



20mM. It is not yet known if the two systems are independent or simply alternative forms of the same mechanism, but it is clear that the hexose kinases are involved in the high affinity system and that this system is subject to glucose repression (Bisson and Fraenkel, 1984). Since similar high and low affinity systems have been demonstrated for 2-deoxyglucose (Bisson and Fraenkel, 1983b) it is unlikely that the role of the kinase in the high affinity system is simply to maintain a concentration gradient by promoting the rapid metabolism of substrate. Considering this, alternative explanations based on either the direct interaction of kinase with the transport protein or an indirect phosphorylation independent enzymic activity for the kinases have been offered.

In summary, it is unknown whether there is a single facilitative diffusion uptake augmented by kinase involvement, or two independent diffusion mechanisms, one of which is totally dependent upon kinase.

Because of the involvement of kinases in the uptake of glucose it is difficult to measure the contribution and activity of the transport protein in isolation. Consequently, the results of Figures 2.6 and 2.7 refer to the intracellular accumulation of glucose derived label and are therefore a measure of the sum of the system rather than its individual parts. The observed  $K_m$  for accumulation of 1mM (Figure 2.6) is similar to the 1-2mM value obtained for the high affinity uptake system of Bisson and Fraenkel (1983a). Due to the complexity of glucose transport it is difficult to draw specific conclusions about the results

presented in Figure 2.7. Clearly glucosamine is capable of reducing the accumulation of label, but indicating the point, or points of action for glucosamine is impossible. The failure of the results to fit the standard kinetic plots of inhibitors is probably a reflection of the complexity of uptake. While expecting glucosamine to give results consistent with a competitive inhibitor in simple systems, the fact remains that glucosamine is both transported and phosphorylated indicating that it is a second substrate rather than an inhibitor.

Figure 2.7 suggests that glucosamine is capable of inhibiting accumulation to a maximum of 50%. Since one of the characteristics of competitive inhibitors is that as their concentration rises, the activity of the inhibited protein tends to zero, it is possible that glucose is entering the cell via some uptake system resistant to glucosamine. This would imply at least two uptake systems and is consistent with the view of glucose transport as a multi event mechanism.

The ability to study hexokinase independent of transport allows stronger conclusions to be reached. For glucosamine to be an effective inhibitor of hexokinase it was necessary to demonstrate a  $K_i$  value similar to the  $K_m$  of hexokinase for glucose. The values of 0.19mM and 1.7mM for  $K_m$  and  $K_i$  respectively, are sufficiently similar to support the observation that glucosamine can act as a competitive inhibitor of hexokinase. The evidence presented indicates that glucosamine inhibits both the accumulation of glucose and the activity of hexokinase. It is possible that the effects on accumulation

occur solely as a result of the in vivo inhibition of hexokinase.

However, the structural similarities of glucose and glucosamine, the evidence that glucosamine accumulates intracellularly, as it must if hexokinase is inhibited, (Burger, and Hejmova 1961), and the observation that 2-deoxyglucose has uptake systems identical in nature to those of glucose, makes it unlikely that glucosamine does not enter via the facilitative diffusion pathway of glucose.

Evidence has been presented describing the effect of glucosamine on the ability of yeast to take up and metabolise glucose. Although certain strains of yeast show a resistance to glucosamine, the principle of competition by a non-metabolisable catabolite repressor can be extended to alternative glucose analogues. The controlled reduction of growth rate allowed by this system may bring the convenience of the shake flask to experiments that previously demanded a glucose limiting chemostat.

#### CONCLUSIONS

- 1) Glucosamine concentrations above 0.1% prevent growth on non-fermentable carbon sources.
- 2) Glucosamine increases the population doubling time of cells metabolising glucose. This reduction occurs in a predictable manner and is determined by the absolute concentration of glucose and the glucose:glucosamine ratio.
- 3) Although glucosamine reduces the growth rate of glucose grown cells, its ability to mimic glucose repression ensures that cells remain catabolite repressed.
- 4) Glucosamine inhibits the accumulation of labelled glucose.

5) Glucosamine competes with glucose, acting as a competitive inhibitor of hexokinase.

CHAPTER 3  
SUPPRESSORS  
OF cdc39

SUPPRESSORS OF cdc39INTRODUCTION

The traditional and most informative method of elucidating steps in a pathway is to identify the components and their temporal relationships. Saccharomyces cerevisiae is an organism that lends itself readily to the isolation of mutants providing that reasonable selection protocols exist. Hartwell, Culotti and Reid, (1970), were the first to use this approach to examine the cell cycle of yeast. By screening a collection of temperature sensitive mutants they were able to identify strains carrying defects that resulted in a characteristic terminal phenotype at the restrictive temperature. Analysis of this class of mutants enabled them to produce a temporal map ordering the mutants with respect to phenotypic landmarks throughout the cycle (Hartwell et al. 1974). The resulting map demonstrated that the cell cycle was composed of two independent pathways, one leading to the initiation of DNA synthesis, the other to bud emergence. It further demonstrated that the product of the CDC28 gene was needed for both. They realised that if a single event were to halt both dependency pathways, it would have to occur at a point common to both. This, along with the observation that cdc28 mutants had a G1 arrested phenotype, indicated that the CDC28 gene product may have an important role in cell cycle regulation. Since completion of the CDC28 mediated step was necessary to allow the cell to leave G1 and enter S phase, this event was termed "start".

The observation that the CDC28 gene step is interdependent

with alpha factor arrest and that cdc28 arrested cells retained competency to mate, allowed Reed to design a protocol that would select further "start" mutants (Reed, 1980). This approach identified three new "start" genes CDC36, CDC37 and CDC39. Bedard, Johnston and Singer, (1981), were able to isolate further "start" mutants using a protocol that selected mutations protecting cells containing the cdc4 and cdc7 mutations, from the inviability that accompanied these lesions. Following the isolation of these "start" mutants, much work is being done on the characterisation of their gene products. It is hoped that this approach will allow an understanding of cell cycle regulation at the biochemical level (Reed, 1984).

Given the potential complexity of "start" it is possible that other "start" genes exist but cannot be selected by the procedures thus used. Indeed, it is conceivable that they cannot be isolated in a conditional form. Attempts have been made to isolate new "start" genes by identifying extragenic suppressors of the temperature sensitive phenotype shown by those already available (Reed, pers. comm). This type of search could turn up two interesting classes of mutant, each of which would imply different things about the nature of the suppressed gene. The first is a mutation that results in a gene product that acquires the ability to supply the missing cdc gene function. These are more likely to be found if genes exist whose wild-type product is similar but not identical to that of the CDC gene. If this is the case it is possible that these gene products may also have regulatory functions analogous to those of the "start" genes

products. The second class of suppressor demands that the "start" gene product functions as part of a multimeric complex, whose integrity is disrupted by the mutation. The fatty acid synthetase complex is an example of such a multimeric complex. The suppressing mutation would be in a second component of the complex, enabling at least a partial restoration of integrity and activity. In this case the suppressor gene should be considered as much a "start" gene as the original CDC mutation, since it functions in the same process.

Although attempts have been made to isolate these suppressors by looking for mutations that will restore the ability to grow at the restrictive temperature (Reed, pers. comm.), none have, as yet, been found. Nevertheless, this approach has been successful with some "non-start" cdc genes (Thomas, Norvick and Botstein, 1984; Pringle, Coleman, Adams, Lillie, Haarer, Jacobs, Robinson and Evans, 1984). The potential of this technique remains, although it may be more successful if the selection procedure is based on a characteristic of the "start" genes other than their temperature sensitivity. The suppression of the G1 arrest of cdc36 and cdc39 mutations by MAT heterozygosity, without suppression of temperature sensitivity, supports this.

It has been reported (Shuster, 1982b) that the temperature sensitivity of strains bearing the "start" mutations cdc36 or cdc39 can be suppressed by growth on a derepressive carbon source. I report here an attempt to isolate suppressors of cdc39 by selecting genetic derepression in a cdc39 background.



## RESULTS

### Carbon Source Effects

The observation that carbon catabolite derepression media are capable of suppressing the temperature sensitive phenotype of certain "start" mutations (Shuster, 1982b) implies that there may be some interaction between catabolite repression or carbon metabolism, and cell cycle regulation at "start". However, the experiments as reported lacked appropriate controls and alternative propositions explaining the suppression of the cdc mutants were possible. It is not difficult to postulate a hypothesis in which the CDC genes produce an enzyme of reduced activity whose product functions independent of growth rate in an all or nothing manner to initiate a new cycle. During the rapid doubling of cells growing on glucose, this activity is insufficient to allow accumulation to a critical concentration of the initiating product. The slower cycle of pyruvate grown cells allows sufficient of this initiator to be produced ensuring that a new cycle follows. For reasons such as this, Shuster indicated that suppression in pyruvate may be a consequence of a reduction in growth rate. He demonstrated that slowing the growth rate using a protein inhibitor did not suppress the cdc mutation. This is an unsuitable control for 2 reasons. A) The reduction in growth rate afforded by this addition did not bring the growth rate down to that of the pyruvate cells. B) The use of a protein inhibitor may have affected other aspects of "start" in an unknown manner. It is clear that although Shusters observations are interesting, any long term study of this

phenomenon must be based on more substantial evidence than that offered. To this end Shusters experiments were repeated using more appropriate controls.

As has been shown, D-glucosamine is capable of reducing the growth rate of cells metabolising glucose without affecting their repression status (Chapter 2). This sugar can therefore be used to produce repressed cells growing on glucose + glucosamine at rates below those of derepressed cells. Figure 3.1 shows that SR665-1 (cdc39) arrests at 36°C on both 2% glucose and 1% glucose + 1.4% glucosamine. The observation that the growth rate increased at 36°C on both pyruvate and acetate, illustrated that the suppression of cdc39 by derepressive carbon sources was independent of both the growth rate and the specific derepressive carbon source. Further experiments were performed to show that suppression of cdc39 was not due to some interaction of catabolite repression with the genetic background of SR665-1 (Figure 3.2).

2-15, a second strain containing the cdc39-1 allele, clearly showed the same properties as SR665-1 with respect to temperature sensitivity and suppression. Figure 3.2 also indicated that SM6, a strain containing the cdc28-6 mutation, could not continue growth at the restrictive temperature when metabolising either glucose or pyruvate. Its temperature sensitive arrest in pyruvate followed the same pattern as that in glucose. In both cases there seemed to be a lag period of 300 minutes before the growth rate was reduced considerably. This lag was significantly longer than that displayed by cells containing the cdc39 mutation

**FIGURE 3.1**      EFFECTS OF CARBON SOURCE ON TEMPERATURE SENSITIVITY  
OF SR665-1 (cdc39)

Open symbols indicate incubation at 23°C, closed symbols incubation at 36°C. The normalised data allow direct comparisons to be made. Data were normalised by cells/ml at t=X divided by cells/ml at t=0. Cells were shifted to 36°C at t=250

(A) = YEP+ 2% glucose

(B) = YEP+ 3% sodium pyruvate

(C) = YEP+ 1% glucose + 1.4% D-glucosamine

(D) = YEP+ 3% sodium acetate

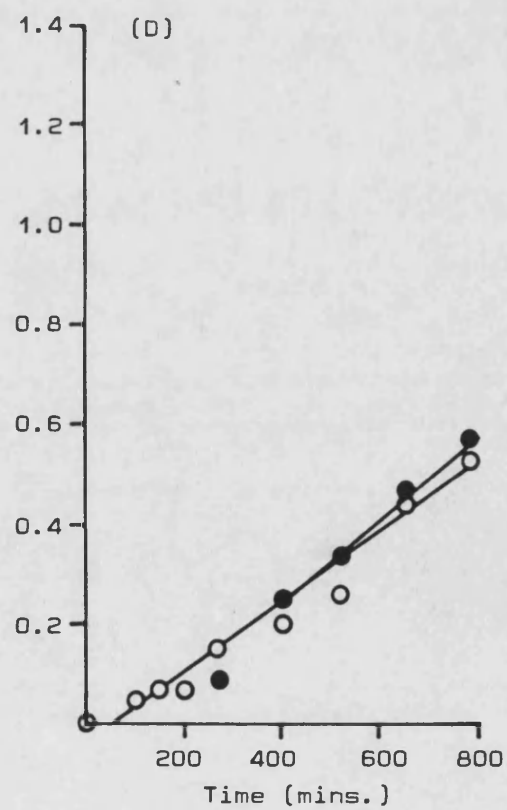
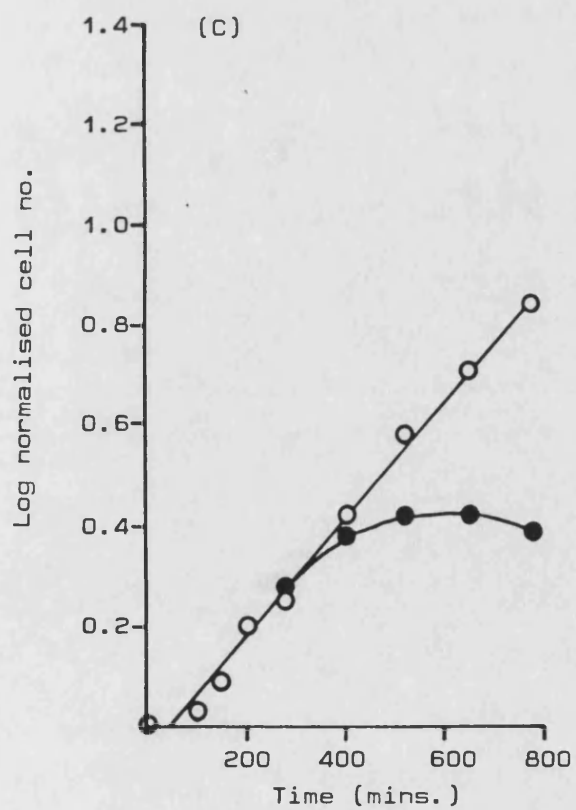
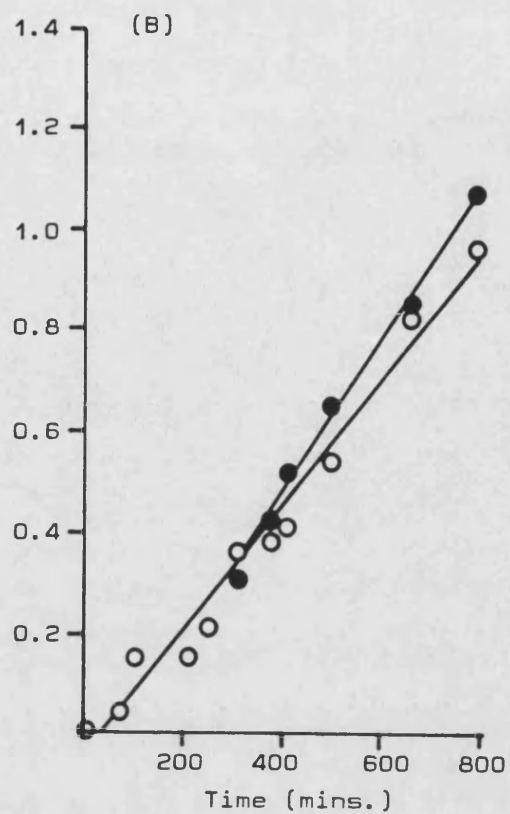
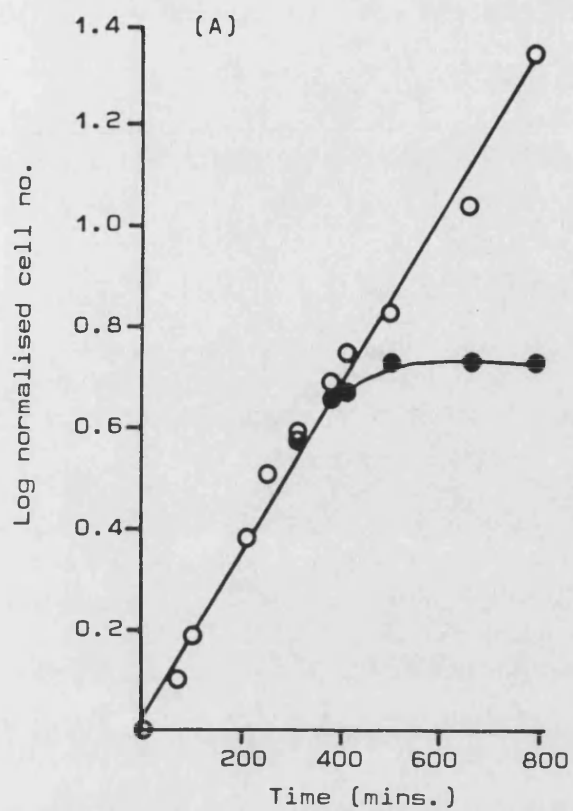
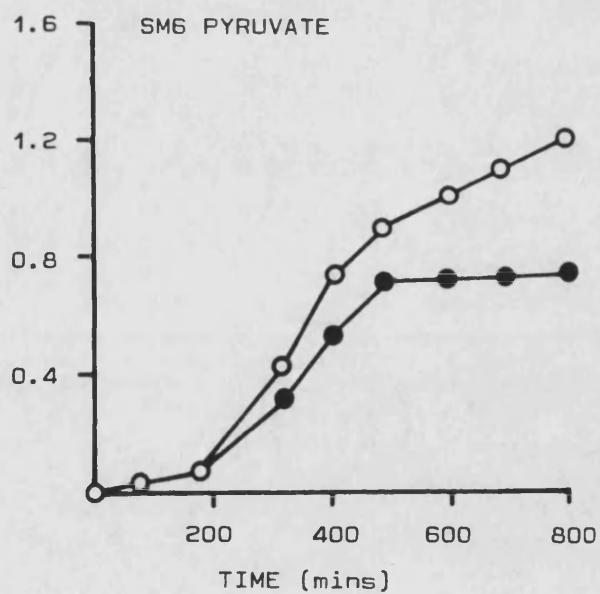
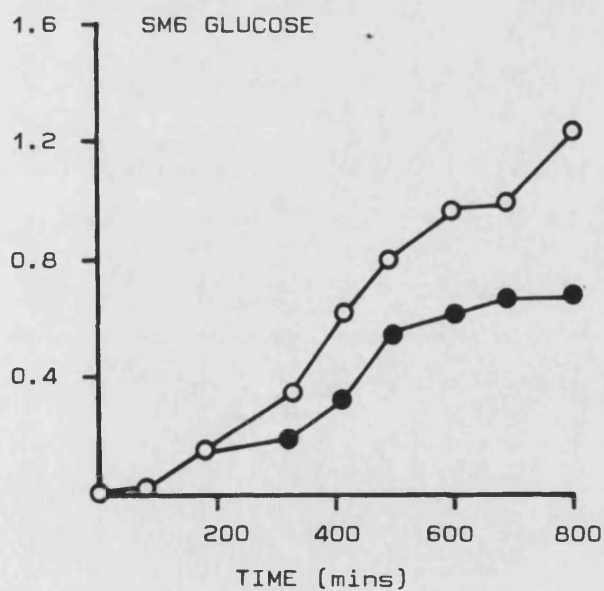
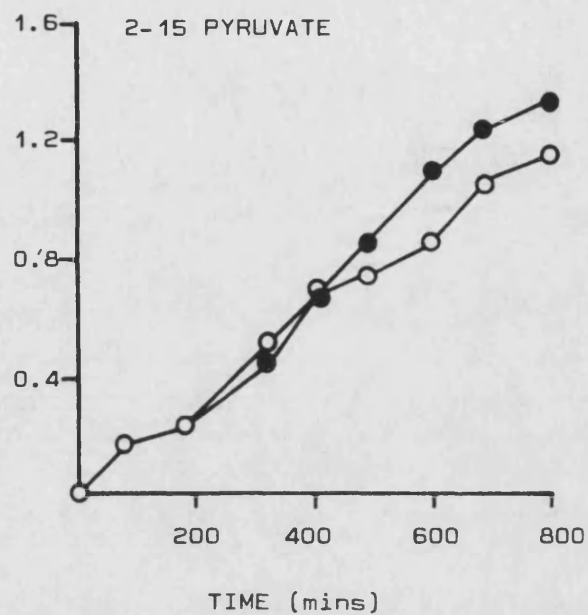
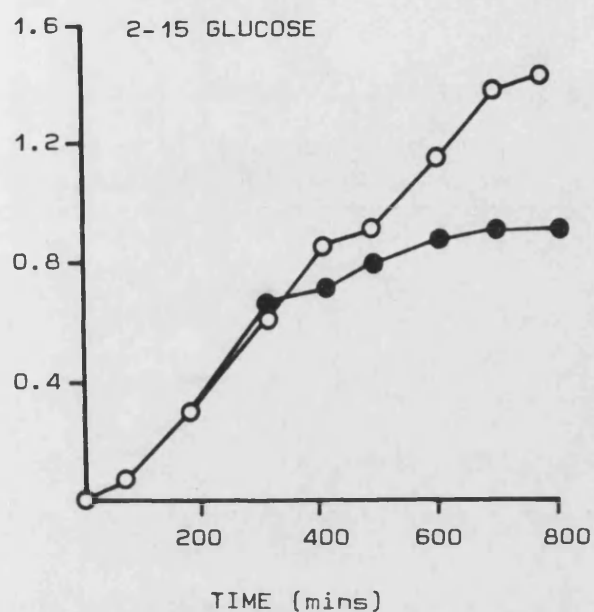


FIGURE 3.2 CARBON SOURCE EFFECTS ON START MUTANTS

Strains containing "start" mutants were grown at 23°C ( ● ) and 36°C ( ● ) on YEP+ 2% glucose or YEP+ 3% sodium pyruvate. 2-15 = cdc39-1, SM6 = cdc28-6. Cells were shifted to 36°C at t=200

The ordinate scale shows log normalised cell number increase (normalisation as described in Figure 3.1).



Ordinate axis in each case = log normalised cell number.

(Figures 3.1 and 3.2). The slow curve seen for SM6 was reminiscent of nutrient starvation arrest. However, in both glucose and pyruvate the maximum cell density at 36°C was only  $4 \times 10^6$  cells/ml. Since the 23°C control indicated that both media were capable of supporting higher densities, it was unlikely to be nutrient arrest.

The results regarding the suppression of cdc39 confirmed those of Shuster. Clearly the observed suppression was independent of growth rate and probably the specific genetic background. Since similar results were obtained on pyruvate or acetate it was also independent of the specific derepressive carbon source. Consequently, it was concluded that the suppression of cdc39 at 36°C on derepressive carbon sources, was the result of an interaction between "start" and some biochemical or morphological feature of derepressed cells.

#### Isolation of Suppressors of cdc39

##### Rationale of selection

It has been demonstrated that some aspect of catabolite derepression is capable of suppressing cdc39. Since catabolite repression/derepression is a complex, poorly understood phenomenon, it is difficult to identify the peculiar aspect of derepression responsible for the suppression of cdc39. The ability of glucosamine and other glucose analogues to induce catabolite repression similar to that of glucose has been used to isolate catabolite repression resistant mutants (Michaels and Romanski, 1980). Several such mutants have been isolated. In each case, the mutation affects a subset of those features

regulated by catabolite repression (Table 1.1).

It was reasoned that if physiological derepression could suppress cdc39, it might be possible to isolate single gene mutations that suppressed cdc39 by affecting that aspect of catabolite repression responsible for suppression. This approach, if successful, could, a) facilitate a study of the interaction between general carbon metabolism and "start" at the genetic level; and b) considerably reduce the range of biochemical changes that need to be studied to define the aspect or aspects leading to suppression at the biochemical level.

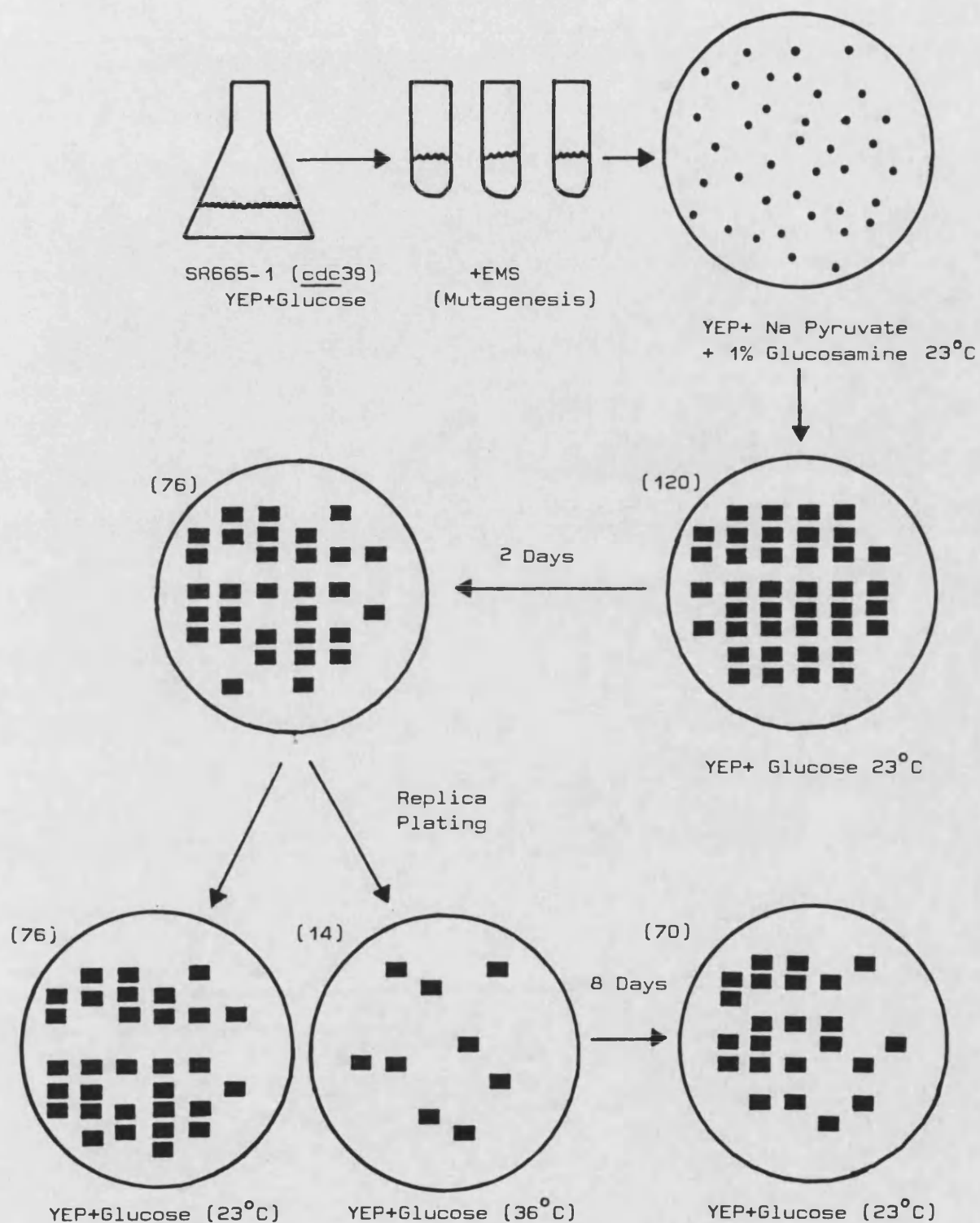
#### Selection of Suppressors

Figure 3.3 presents a diagrammatic representation of the steps taken and results obtained in the isolation of suppressors of cdc39. SR665-1 (cdc39) was grown to mid-exponential phase at 23°C in YEPD. The culture was split and subject to mutagenesis. Following mutagenesis, samples of cells from each of the ten tubes were plated onto YEP+ 3% sodium pyruvate+ 1% glucosamine at 23°C. The presence of glucosamine in this medium represses the cells and prevents their growth using pyruvate as a carbon source. Consequently, only those cells that have acquired one of a range of mutations will be able to form a colony under these conditions. Growth on this medium is extremely slow and demands that cells are incubated for several weeks before screening. At this point several colony types were seen, possibly reflecting the various mutations that may allow growth on the selection medium. Examples of these possible mutations are; A) Exclusion mutants, in which the access of glucosamine is prevented. B)



FIGURE 3.3      SCHEMA DESCRIBING MUTANT ISOLATION

Following mutagenesis, resistance to D-glucosamine was used to select putative carbon catabolite repression resistant mutants in a cdc39 background. These were subsequently tested for simultaneous acquisition of temperature resistance. Bracketed numbers refer to the number of colonies growing at each step.



Deaminase mutants that have acquired the ability to cleave the amine group allowing the cells to metabolise the resulting glucose molecule. If these mutants exist, the absence of fast growing colonies would suggest that the deamination occurs at very low rate; C) Catabolite repression resistant mutants in which glucosamine enters the cell, remains intact, but is unable to prevent the utilisation of sodium pyruvate.

To ensure that the required mutation was not inadvertently overlooked, 120 colonies of various colony types were selected and transferred to YEPD at 23°C. The colonies were picked from plates at all concentrations of EMS but particular emphasis was placed upon selection from those mutagenised with the lowest effective concentrations of EMS. In this way it was felt that the chances of multiple mutations would be reduced. By limiting mutagenesis to one hour prior to selection, the probability was increased that each colony derived from a single independent mutation and was not the result of a division process.

Of the 120 colonies transferred to YEPD at 23°C, only 76 managed to sustain growth over 2 days. This step had two purposes; A) To provide healthy colonies for a subsequent replica plating. B) To reject glucosamine uptake deficient mutants. The similarities between glucose and glucosamine suggested that those colonies acquiring glucosamine resistance via exclusion would also exclude glucose and be incapable of extensive growth on YEPD, the net result being selection against glucosamine exclusion mutants, a class of mutants thought to be uninteresting.

Having isolated potentially interesting glucosamine

resistant mutants, the second step in the selection procedure was designed to test them for the simultaneous acquisition of temperature resistance. Of the 76 colonies that retained viability on YEPD at 23°C, 14 also grew at 36°C. After 8 days the 36°C plate was shifted down to 23°C, a further 36 colonies reinitiated growth, indicating that their temperature sensitive lesion did not lead to inviability. Of the 120 glucosamine resistant mutants originally isolated, 14 had also acquired temperature resistance. The appearance of this class of mutant as a consequence of two independent mutations would occur with a frequency of less than  $10^{-8}$  (Lobo and Maitra, 1977a). In the light of this, the observed frequency of greater than 1:10 indicated that these two characteristics probably occurred as a result of a single mutation. When streaked for single colonies, 3 of the 14 selected mutants proved incapable of rapid growth at 23°C on YEPD. This type of test was far more stringent than those previously carried out and explains why these colonies had been scored positive on previous crude tests. Consequently, they were discarded and the remaining eleven strains examined in more detail.

#### Characterisation of the Mutants

To ensure that the mutants selected were not contaminants and to examine their nutritional requirements following mutagenesis, the mutants were subject to certain growth tests (Table 3.1). It can be seen that these mutants retain the nutritional requirements of their parental strain (SR665-1), but that their response to temperature differs as expected. Even though tests employing the

MEDIUM	STRAIN												
	SR665	2-15	6	14	15	16	18	20	21	22	25	27	28
YEPD (23°C)	3	3	3	3	3	3	3	3	3	3	3	3	3
YEPD (36°C)	0	0	2	3	2	2	3	3	2	1	2	2	1
YEP-P-G	0	0	1	1	2	2	2	2	2	1	1	2	2
YEPD+CYH	2	0	2	2	2	2	2	2	2	2	2	2	2
YMM+SUP-HIS	2	2	2	2	2	2	2	2	2	2	2	2	2
YMM+SUP-MET	0	2	0	0	0	0	0	0	0	0	0	0	0
YMM+SUP-TYR	0	2	0	0	0	0	0	0	1	0	0	0	0
YMM+SUP	2	2	2	2	2	2	2	2	2	2	2	2	2
YMM+TYR	0	0	0	0	0	0	0	0	0	0	0	0	0
YMM+MET	0	0	0	0	0	0	0	0	0	0	1	0	0

TABLE 3.1 GROWTH TESTS ON THE MUTANTS

The figures refer to total growth on plates judged after fixed times relative to appropriate controls. Positive controls are scored as 3, negative controls are scored as 0. The figures in the table indicate the degree of growth within these parameters.

YEP-P-G = YEP+ sodium pyruvate + glucosamine. CYH = cycloheximide.

HIS = histidine. MET = methionine. Tyr = tyrosine. SUP = all necessary supplements.

multipoint inoculator are crude, differences between the mutants in terms of their responses were beginning to emerge, with mutant strains 14, 18 and 20, growing faster than all others. To further characterise the response of the mutants to temperature, their growth was assayed at 23°C and 36°C in repli-dish wells with appropriate strains as controls. At 23°C the growth of the mutants was generally as good as either control. At 36°C the situation was very different. Although a spectrum of patterns of growth were obtained, they fell into three broad categories. Rather than plot the data for all eleven mutants, representatives of the groups have been selected and plotted in Figure 3.4. The mutants grouped as follows; A) Fast growth: mutants 14, 18 and 20. B) Intermediate growth: mutants 16, 15, 16, 21, 25 and 27. C) Poor growth: Mutant 28. The figure indicates that the fast growing mutants have a curve very similar to that of the CDC39 strain, indicating complete suppression of cdc39. The poor growth mutant behaves little better than the cdc39 control. The intermediate group clearly continue growth at the restrictive temperature. In these cases the mutants seem to give partial suppression of the temperature sensitivity of the cdc mutation. The data from these broth cultures largely agrees with the less stringent plate tests (Table 3.1).

Reed has indicated that the suppression of cdc39 by derepressive carbon sources was partial. He stated that the result of growth on pyruvate is to increase the restrictive temperature and cause random arrest rather than the specific G1 arrest of cdc39 cells in YEPD at 36°C (Reed, 1984). Table 3.2

FIGURE 3.4 GROWTH OF SELECTED MUTANTS

Using repli-dishes, the growth of selected mutants and appropriate controls was examined at 36°C in YEPD. The results have been normalised to allow a direct comparison between the different curves (normalisation as described in Figure 3.1).

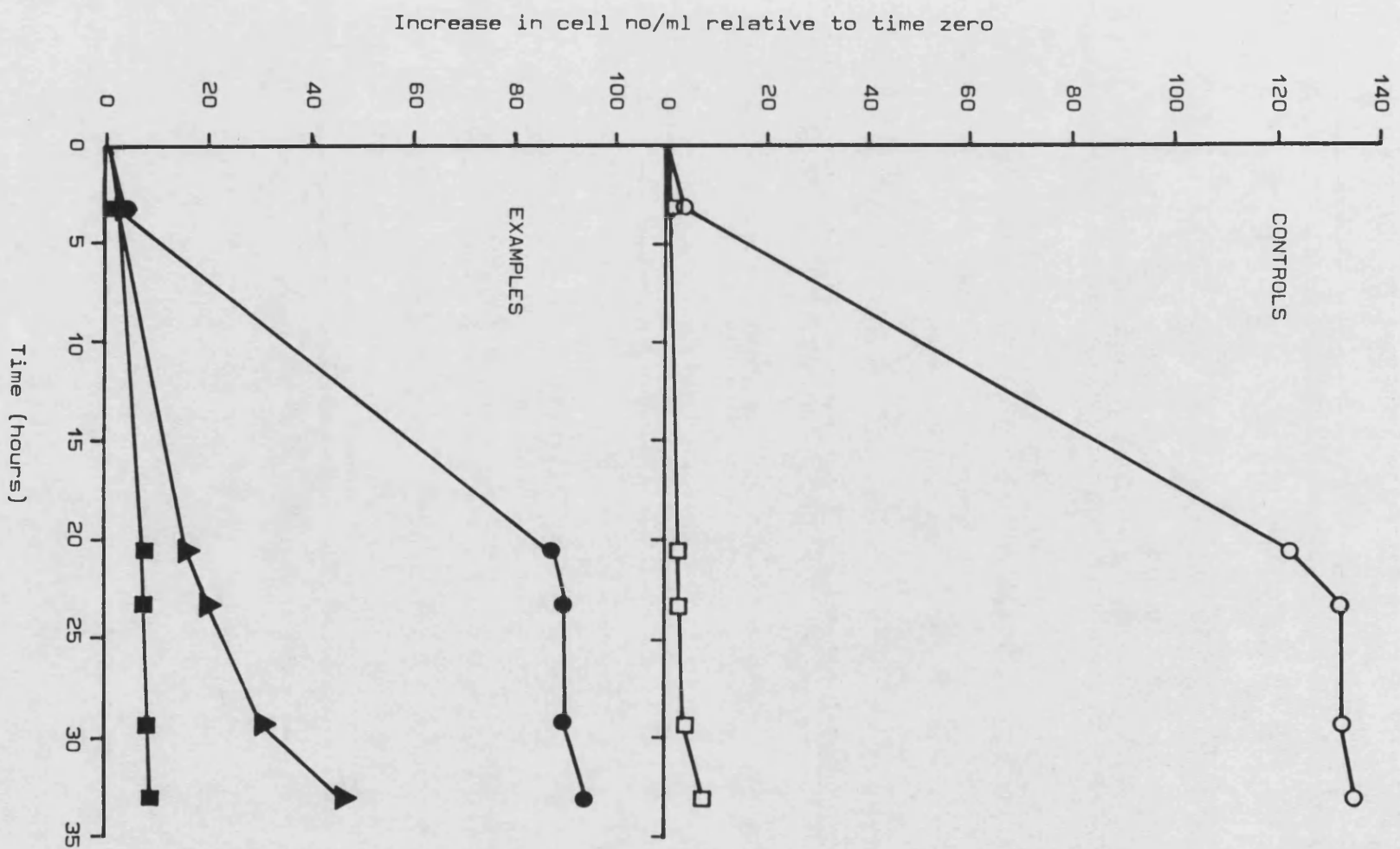
( ○ ) SMC-1B (CDC39)

( □ ) SR665-1 (cdc39)

( ● ) mutant 18

( ▲ ) mutant 22

( ■ ) mutant 28





STRAIN	GLUCOSE			PYRUVATE		
	23°C	36°C	38°C	23°C	36°C	38°C
Mutant 6	+	+	-	+	ND	+
Mutant 14	+	+	+	+	ND	+
Mutant 15	+	+	-	+	ND	+
Mutant 16	+	+	-	+	ND	+
Mutant 18	+	+	+	+	ND	+
Mutant 20	+	+	+	+	ND	+
Mutant 21	+	+	+	+	ND	+
Mutant 22	+	-	-	+	ND	+
Mutant 25	+	+	+	+	ND	-
Mutant 27	+	+	+	+	ND	+
Mutant 28	+	-	-	+	ND	-
2-15 ( <u>cdc29</u> )	+	-	-	+	+	-
322 ( <u>cdc28</u> )	+	-	-	+	-	-
SR662-1 ( <u>cdc36</u> )	+	-	-	+	+	-
SR672-1 ( <u>cdc37</u> )	+	-	-	-	-	-
SR665-1 ( <u>cdc39</u> )	+	-	-	+	+	-
A364A	+	+	+	+	ND	+
a1	+	+	+	+	ND	-
D273-11a	+	+	+	+	ND	+
S288C	+	+	+	+	ND	+

**TABLE 3.2** EFFECTS OF TEMPERATURE AND CARBON SOURCES

The effect of temperature and carbon source on the growth of "start" mutants, strains isolated as suppressors of cdc39, and several controls.

ND = not done. (+) = growth. (-) = no growth.

presents the results of this test on the mutants and appropriate controls. It confirms Reeds observation that the restrictive temperature has been elevated. The mutants fall broadly into 2 groups, those that continue to grow at the elevated temperature and those that do not. It is not clear what this means, but it may indicate that different forms of suppression are being seen; one similar to carbon source suppression, the other a more substantial suppression that restores a wild phenotype. On the basis of this evidence the mutants can be grouped into three classes. Those that grew well at 36°C and continue to grow at 38°C (mutants 14, 18 and 20). Those with intermediate growth rates at 36°C and continued growth at 38°C (mutants 21, 25 and 27), and those that grew at intermediate rates at 36°C but failed to proliferate at the elevated temperature (mutants 6, 15, 16 and 22).

A result not indicated by Table 3.2 was that the growth of the mutants on sodium pyruvate at 36°C was significantly better than that of the parent. Considering the method of their selection, it was difficult to understand how a putative derepression mutation could confer growth advantages over the parent under conditions in which both were derepressed. This may be considered an indication that the mutation did not simply lead to a state of derepression.

At this point it was decided that further physiological and genetical characterisation would only be possible on a few of the mutants. To this end work continued with those mutants giving the best suppression of cdc39 (mutants 14, 18 and 20).

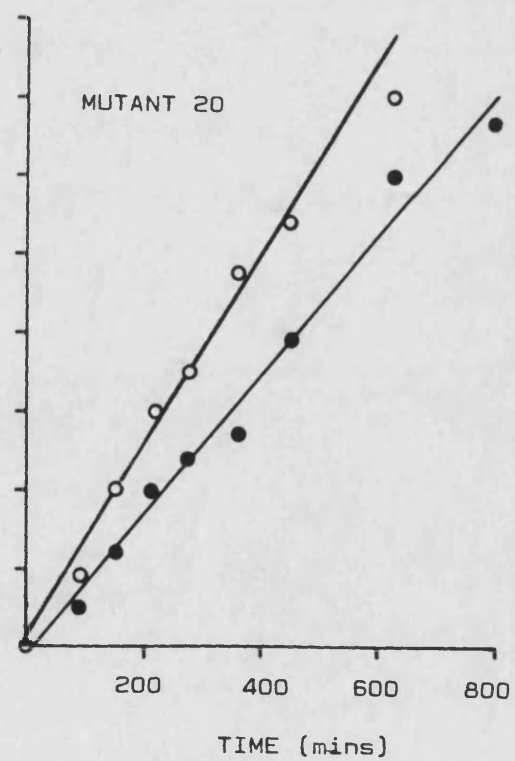
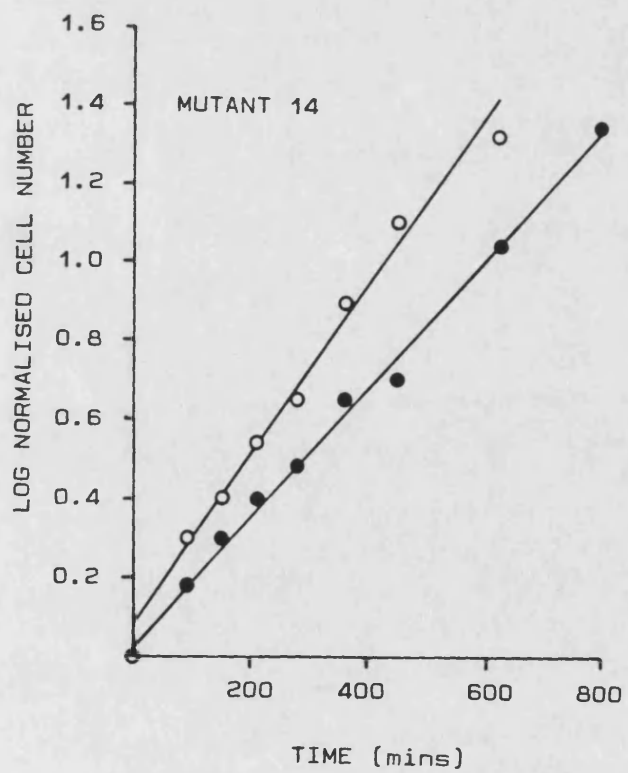
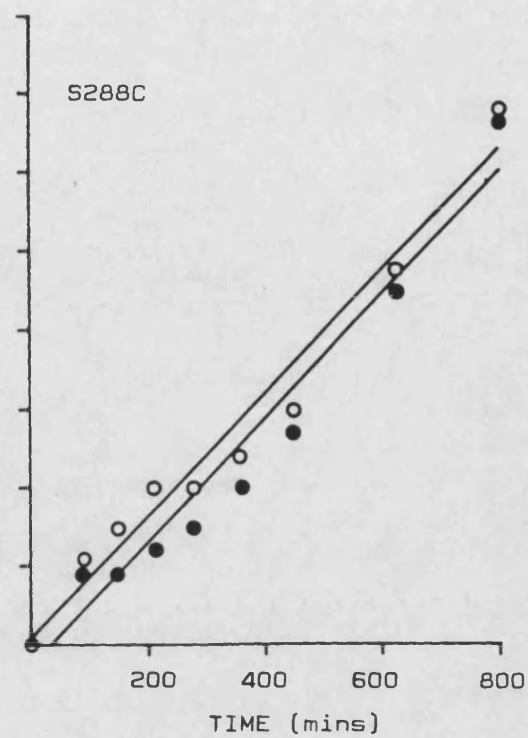
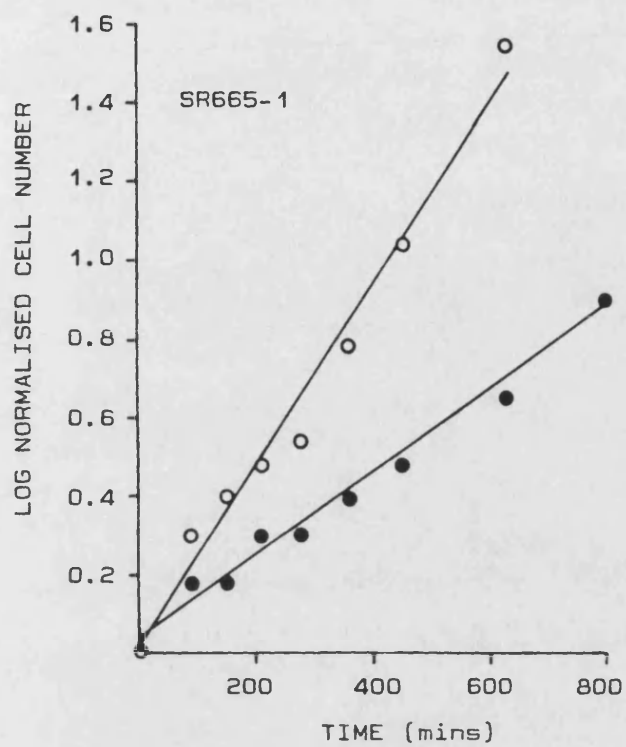
The effect of glucosamine on the growth rates of mutants 14 and 20 is shown in Figure 3.5. SR665-1, the parental strain, showed a marked sensitivity to glucosamine, and although both mutant strains retained a sensitivity to glucosamine it was relatively much reduced. Nevertheless, their continued sensitivity indicated that they did not exclude glucosamine. The reduction in sensitivity may be a manifestation of the mechanism by which they were resistant to glucosamine. The control strain S288C was clearly insensitive to glucosamine, the addition to cells growing on glucose having no effect on the growth rate.

#### Invertase Activity

Invertase (B-D-fructofuranoside fructohydrolase EC.3.2.1.26) catalyses the hydrolysis of sucrose to glucose and fructose, and is subject to glucose regulation of its synthesis. It has frequently been used as an indicator of the cells repression status (Bailey and Woodward, 1984; Entian and Mecke, 1982; Michels, Hahmengerger and Sylvestre, 1983). Several carbon catabolite repression resistant mutants have high levels of invertase in the presence of glucose (hex2, grr1 and glr1). If the selected mutants suppress cdc39 by genetically conferring resistance to catabolite repression, it would be predicted that they too would lose the glucose regulation of their invertase synthesis. Saccharomyces cerevisiae contains two invertase isoenzymes. The larger form is bound external to the cytoplasmic membrane and is sensitive to glucose repression. Under repression conditions all invertase is found as a smaller intracellular form (Gascon and Lampen, 1968). As a consequence

FIGURE 3.5 THE EFFECT OF D-GLUCOSAMINE ON SELECTED MUTANTS

The effects of D-glucosamine on the growth rate of temperature resistant mutants isolated from SR665-1 (cdc39), was assayed by comparing growth in YEPD ( ○ ) and YEPD+ 1% D-glucosamine ( ● ). Normalisation was carried out as described in Figure 3.1.



it was thought that the effects of repression would be different in the soluble and insoluble fractions of a cell preparation. Table 3.3 presents the results of invertase assays on mutants 14 and 20 with those of appropriate controls. The similarity between the results of the soluble and insoluble fractions probably indicates that the method of preparation solubilized the bound form. Gascon and Lampen (1968) have reported that the large iso-enzyme can be released by sonication. Consequently, the results presented for either fraction may represent total invertase activities.

F6 is a wild-type control. The invertase activity was lower in both fractions of the glucose grown cells than those of the pyruvate grown cells. This trend was not seen in strain DGX1-3A, which contains the grr1 mutation and is resistant to glucose regulation of its invertase synthesis (Bailey and Woodward, 1984). Invertase activity in this strain was significantly higher than in both F6 and the mutants. The invertase activity of the mutants was higher in all cases in cells grown on pyruvate than those of glucose. With respect to the insoluble portion they showed a pattern that resembled F6 much closer than DGX1-3A.

In the soluble portion they demonstrated a marked lack of stimulation in the absence of glucose. Although these mutants were initially isolated using the protocol that picked up the grr1 strain, there was no evidence to suggest that either of the mutants was resistant to the catabolite repression of invertase. Since different derepression genes affect different enzyme systems (Table 1.1), it was possible that the mutants contained

STRAIN	SOLUBLE FRACTION		INSOLUBLE FRACTION	
	GLUCOSE	PYRUVATE	GLUCOSE	PYRUVATE
DGX1-3A	$1.6 \times 10^{-2}$	$5.3 \times 10^{-2}$	$2.6 \times 10^{-2}$	$2.6 \times 10^{-2}$
F6	$2.4 \times 10^{-3}$	$3.9 \times 10^{-2}$	$6.8 \times 10^{-3}$	$5.0 \times 10^{-2}$
MUT 14	$2.3 \times 10^{-3}$	$5.8 \times 10^{-3}$	$7.0 \times 10^{-3}$	$2.0 \times 10^{-2}$
MUT 20	$2.0 \times 10^{-3}$	$8.1 \times 10^{-3}$	$5.8 \times 10^{-3}$	$2.5 \times 10^{-2}$

TABLE 3.3      INVERTASE ACTIVITIES

Average invertase activities ( $\mu\text{m}$  glucose/min/ $\mu\text{g}$  protein) from cells growing under repressed and derepressed conditions. Samples were taken at 2 hour intervals from exponentially growing populations.

elements of constitutive derepression for enzymes other than invertase. However, since invertase seemed to be affected in all the repression mutants isolated to-date this was unlikely. It was also possible that invertase derepression mutants were originally picked up but that they were inadvertently rejected during subsequent selections.

#### GENETICS OF THE MUTANTS

To characterise the mutants designated initially as cdc39, X-, they were crossed to S.cerevisiae 2-15 (cdc39). This cross allowed a study of dominance relationships in the diploid and should have allowed the suppressor X-, to be crossed out of the SR665-1 background. Putative diploids were selected on the appropriate medium and transferred to sporulation agar. Concomitant with this, tests on the properties of the diploids were carried out. These diploids failed, even after several days, to sporulate. The failure to sporulate raised doubts about the ploidy of the strains that had been selected as being diploid. However, it had previously been reported that strains isolated as constitutive derepression mutants were sporulation deficient (Montenecourt, Kuo and Lampen, 1973). To ensure that the strains were sporulation deficient diploids, their ploidy was tested using sst indicator strains. The sst strains carry a mutation (sst2-1) that makes them supersensitive to the mating pheromone secreted by cells of the opposite mating type (Chan and Otte, 1982a; Chan and Otte, 1982b). On a sst lawn this supersensitivity manifests itself as a halo around colonies



secreting the appropriate mating pheromone (Figure 3.6).

The results of these tests and those examining dominance relationships are shown in Table 3.4. From this it can be seen that MATa strains 2-15 and a1 inhibited the growth of the MAT $\alpha$ , sst2-1 lawn. The MAT $\alpha$  strain D273-11a inhibited the MATa, sst2-1 lawn. As expected the diploid SR665-1/F6 inhibited neither strain. Mutant strains 14 and 20 behave like SR665-1 indicating them all to be of the MAT $\alpha$  mating type. The 4 putative diploids from crosses between mutant and 2-15 (designated 215/SR-14-1 to 215/SR-20-4) were incapable of inhibiting the growth of either sst lawn. This indicated that like diploids, they failed to produce mating pheromone. Although this was not conclusive proof that they were diploids, it was unlikely that they were not, since the haploid mutants had already been shown to secrete alpha-factor. The control cross between 2-15 and SR665-1 was carried out to investigate if sporulation deficiency was a property of the suppressor mutation. Initially, it was thought that this too was sporulation deficient. However, by following several sporulation regimes it was possible to induce low frequency sporulation in several crosses. The sporulation frequencies observed were, 215/SR-14-4=0.3%, 215/SR-20-3=1.6% and 215/SR665-1=1.6%. In these crosses both linear and normal asci were observed. Sporulation was not seen in any of the other crosses. From these data it was concluded that the sporulation defect was independent of the cdc39 suppressor and that it was likely to be intrinsic to either SR665-1 or 2-15.

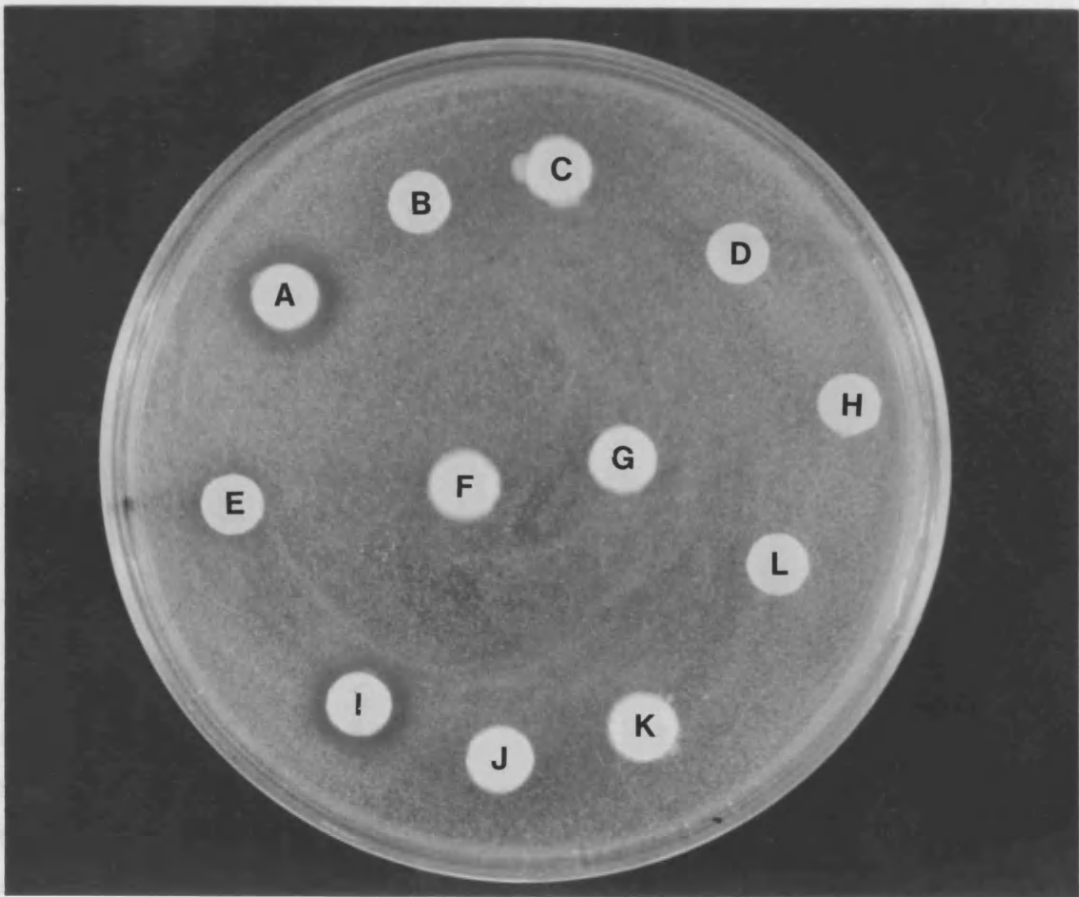
The dominance relationships described in Table 3.4 are

FIGURE 3.6      MATING-TYPE TEST

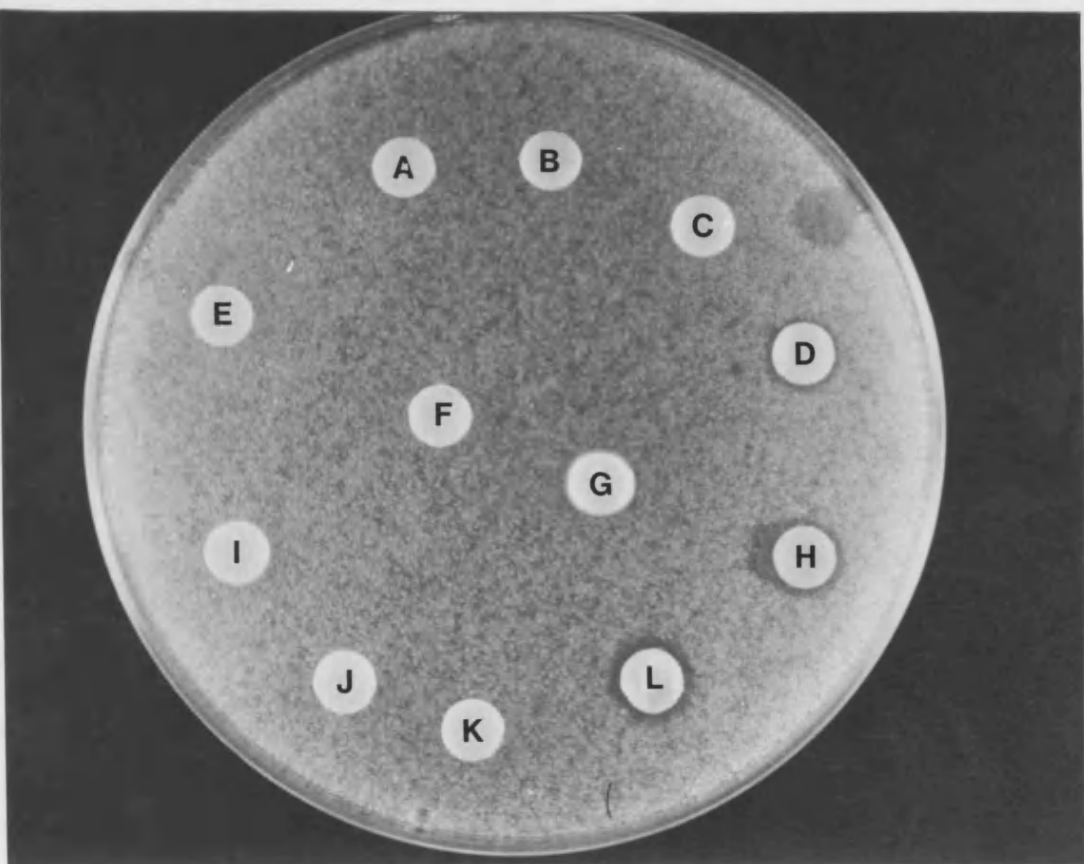
Strains carrying the sst2-1 mutation are supersensitive to the mating pheromone secreted by cells of the opposite mating-type. When spread as a lawn this sensitivity can be used to indicate the mating-type of test strains. Paper discs were soaked in overnight cultures of the strains to be tested, then placed onto MAT $\alpha$  (RC631, photograph A), and MAT $\alpha$  (RC757, photograph B) lawns.

A= 2-15,      B= 215/SR-14-1,      C= 215/SR-20-1,      D= SR-14,  
E= SR665-1/F6,      F= 215/SR-14-2,      G= 215/SR-20-2,      H= SR665-1,  
I= a1,      J= 215/SR-14-3,      K= 215/SR-20-3,      L= SR-20.

A



B



STRAIN	YEPD 23°C	YEPD 36°C	YEP-P-G 23°C	INHIBITION OF <u>sst2-1</u> STRAINS	
				<u>MAT<sub>a</sub></u>	<u>MAT<sub>α</sub></u>
215/SR-14-1	+	+	-	-	-
215/SR-14-2	+	+	-	-	-
215/SR-14-3	+	+	-	-	-
215/SR-14-4	+	+	-	-	-
215/SR-20-1	+	+	-	-	-
215/SR-20-2	+	+	-	-	-
215/SR-20-3	+	+	-	-	-
215/SR-20-4	+	+	-	-	-
SR-14	+	+	+	+	-
SR-20	+	+	+	+	-
SR665-1	+	-	-	+	-
2-15	+	-	-	-	+
a1 ( <u>MAT<sub>a</sub></u> )	ND	ND	ND	-	+
D273-11a ( <u>MAT<sub>α</sub></u> )	ND	ND	ND	+	-
SR665-1/F6 (a/α)	+	+	-	-	-

**TABLE 3.4**      **DIPLOID CHARACTERISTICS**

Characterisation of putative non-sporulating diploids derived from crosses between mutants 14 & 20 and SR665-1. Strains containing the sst2-1 mutation are supersensitive to mating pheromone. Failure to grow in the presence of pheromone from cells of the opposite mating type results in a halo of inhibition and is indicated by a (+).  
 (-) = no growth.    ND = not done.

difficult to explain. Failure of the diploids to grow at 23°C on YEP + pyruvate + glucosamine (YEP-P-G), suggested that the mutation conferring resistance to glucosamine was recessive to its wild-type allele. The continued growth of diploids at 36°C indicated that the suppressor of cdc39 was dominant to its wild-type allele. This observation made the relationship between the suppressor of cdc39 and glucosamine resistance difficult to explain within a single gene model. The evidence suggested that more than one gene was involved.

During the course of a concurrent study, strain F6 was demonstrated to mate well with SR665-1 giving diploids that sporulated with high frequency. It was believed that by following the segregation of temperature resistance and glucosamine resistance, information concerning the number of genes involved and their linkage would be obtained. F6 is a glucosamine sensitive, CDC39 strain that grows well at 36°C. This strain was crossed to mutants 14, 18 and 20 as well as SR665-1. The diploids sporulated well at frequencies of about 70%. The resulting tetrads were dissected and the recombinant progeny subjected to analysis. It was found that replica-plating or multi-point inoculation gave variable results that included many false positives. Consistent, reproducible data were only obtained when a "stringent" test was employed. This test demanded that all strains were streaked for single colonies. Those strains unable to form individual colonies were scored negative. In this way potential errors from cross-feeding or inoculum dependency were circumvented. However, even though the

stringent test was found to give the best results, intermediate levels of growth occasionally made scoring very difficult. This is best illustrated by Figure 3.7.

If we assume that the selected suppressors of cdc39 are NOT capable of suppressing cdc39, a cross between any of the mutants and F6 should yield asci in which temperature sensitivity and temperature resistance segregated 2:2. At the other extreme, were the suppression of temperature sensitivity caused by reversion of the original cdc39 mutation, no temperature sensitive spores should be found. Table 3.5 presents the results of the tetrad analysis on these crosses. The upper section indicates that the majority of asci from the crosses contained a single temperature sensitive spore. Recovery of this temperature sensitive spore indicated that the mutants still contained a temperature sensitive gene capable of preventing growth at 36°C, and that its suppressor was extragenic. The second temperature sensitive allele in each of the asci is probably masked by the suppressor acting epistatically. F6 x SR665-1, gave the 2:2 segregation expected. This control cross illustrated two important points. The first was that F6 itself could not supply a gene capable of suppressing cdc39. Secondly, the clear 2:2 segregation indicated that F6 probably contained no temperature sensitive alleles and that resistance to temperature gave clear growth at 36°C.

The central section of Table 3.5 contains the data relating to glucosamine resistance. In all cases glucosamine resistance segregated 2:2. This indicated that the phenotype was the result

FIGURE 3.7      STRINGENT TESTS

Scoring temperature resistance and temperature sensitivity was carried out using a "stringent" test. Figure A illustrates how intermediate levels of growth made scoring difficult. Top right hand culture = +. Top left hand culture = -. The bottom left show intermediate growth, scoring + as a thick culture but - as single cells. Figure B shows clear 2:2 (top left) and 4:0 (bottom right) segregations. The remaining plates contain intermediate colonies.

A



B

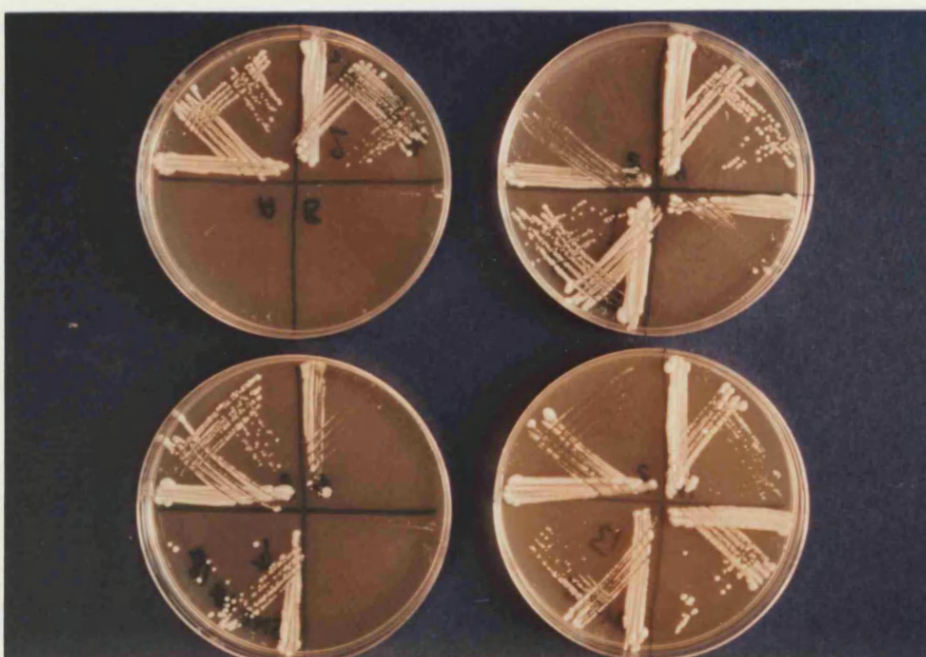




TABLE 3.5      TETRAD ANALYSIS OF MUTANTS

Strain F6 was crossed with SR665-1 and mutants 14, 18 and 20. The diploids were sporulated and the segregation of temperature resistance and glucosamine resistance followed. Only asci in which 4 spores germinated were scored. Following tetrad dissection the recombinant haploids were streaked onto appropriate media. Those unable to form single colonies were scored as negative. The upper table describes the segregation of temperature resistance assayed at 36°C on YEPD. The segregation of glucosamine resistance, assayed by growth on YEP + pyruvate + glucosamine, is shown in the middle table. The co-segregation of temperature resistance and glucosamine resistance is indicated by the lowest table. The numbers refer to single spores from the asci in the upper tables. TR = temperature resistant. TS = temperature sensitive.

CROSS	No OF ASCI SHOWING TR:ts RATIO			TOTAL No SPORES		RATIO TR:ts
	4:0	3:1	2:2	TR	ts	
F6xMUT 14	1	3	0	13	3	4:1
F6xMUT 18	0	4	1	14	6	2:1
F6xMUT 20	3	2	0	18	2	9:1
F6xSR665-1	0	2	10	26	22	1:1

CROSS	No OF ASCI SHOWING GR:gs RATIO			TOTAL No SPORES		RATIO GR:gs
	4:0	3:1	2:2	GR	gs	
F6xMUT 14	0	0	4	8	8	1:1
F6xMUT 18	0	0	5	10	10	1:1
F6xMUT 20	0	0	5	10	10	1:1
F6xSR665-1	0	0	0	0	48	0:48

CROSS	SEGREGATION OF TEMPERATURE RESISTANCE AND GLUCOSAMINE RESISTANCE			
	TR,GR	TR,gs	ts,GR	ts,gs
F6xMUT 14	6	7	2	1
F6xMUT 18	8	6	2	4
F6xMUT 20	8	10	2	0
F6xSR665-1	0	26	0	22

TABLE 3.5      TETRAD ANALYSIS OF MUTANTS

of a single mutation. As expected, the SR665-1 X F6 cross generated no glucosamine resistant spores. This observation confirmed that the glucosamine resistance marker had been acquired by the mutants.

If temperature resistance and glucosamine resistance were the result of a single mutation, one would expect these features to co-segregate. Indeed, if glucosamine resistance were the cause of the temperature resistance then temperature sensitive, glucosamine resistant strains should never be seen. This phenotype appeared in all the crosses and was strong evidence that the two resistance markers were the result of different mutations. Using the observed values, it was possible to compare the results obtained with those that either of two explanations would predict. One model states that both glucosamine resistance and temperature resistance are the result of a single mutation. The mutants in this case would be genetically described as cdc39, X-. The second model states that the two features are independent. In this case the mutants would be, cdc39, X-, Y- with X- leading to suppression of temperature resistance and Y- conferring glucosamine resistance.

Table 3.6 contains this comparison. The observed values are those of phenotype, the expected values were determined using the predicted phenotype of those genotypes described in column two. As has been discussed, the one gene model eliminated the possibility that temperature sensitive, glucosamine resistant (ts,GR) segregants would be generated. Since these had been observed this model was abandoned. The predictions of the two

PHENOTYPE	GENOTYPE	MUTANT						MODEL	
		14		18		20		2 GENES	1 GENE
		No.	%	No.	%	No.	%	%	%
TR,GR	<u>CDC39</u> , <u>X</u> <sup>-</sup> , <u>Y</u> <sup>-</sup>	6	37	8	32	8	32	37	50
	<u>CDC39</u> , <u>X</u> <sup>+</sup> , <u>Y</u> <sup>-</sup>								
	<u>cdc39</u> , <u>X</u> <sup>-</sup> , <u>Y</u> <sup>-</sup>								
TR,gs	<u>CDC39</u> , <u>X</u> <sup>+</sup> , <u>Y</u> <sup>+</sup>	7	44	6	24	10	40	37	25
	<u>CDC39</u> , <u>X</u> <sup>-</sup> , <u>Y</u> <sup>+</sup>								
	<u>cdc39</u> , <u>X</u> <sup>-</sup> , <u>Y</u> <sup>+</sup>								
ts,GR	<u>cdc39</u> , <u>X</u> <sup>+</sup> , <u>Y</u> <sup>-</sup>	2	13	2	8	2	8	13	0
ts,gs	<u>cdc39</u> , <u>X</u> <sup>+</sup> , <u>Y</u> <sup>+</sup>	1	6	4	16	0	0	13	25
CHI <sup>2</sup> VALUE		0.083		0.53		2.13			

TABLE 3.6

## SEGREGATION OF CHARACTERISTICS FROM MUTANTS

After crossing mutants 14, 18 and 20 to F6, the diploid was sporulated and the resulting asci subject to tetrad analysis. The segregation of temperature resistance and glucosamine resistance was followed. The observed segregations were compared with the expectations of a one gene or two gene model (for description of models see text).

The  $\chi^2$  values are based on the predictions of the two gene model and were determined using Yates correction.

gene model were in good agreement with the observations. While this simple analysis did not prove the two gene model, it was strongly supportive. Given the high correlation with a model based on the random segregation of cdc39, X- and Y- it is unlikely that any of these genes were tightly linked.

The conclusions from this phase of the genetic characterisation were that. A) Mutants 14, 18 and 20 contained an extragenic suppressor of cdc39 as judged by the ability to recover temperature sensitive recombinants. B) Glucosamine resistance segregated in the manner of a single mutation and was unlinked to the suppressor of cdc39.

Since there should have been two cdc39 alleles in each of the asci scored 3:1, it was reasonable to assume that one of the spores scored temperature resistant, contained both cdc39 and its putative suppressor. Since these two genes were unlinked, it should have been possible to recover the temperature sensitive allele by crossing to appropriate CDC39 strains and examining the haploid progeny. To this end, progeny from an ascus scored as 3:1 TR:ts (F6/14(4)) were crossed with appropriate strains (Table 3.7). F6/14(4A) and F6/14(4D) are both temperature resistant spores which, when crossed to F6, generated only temperature resistant progeny. This indicated that neither they nor F6 contained masked temperature sensitive alleles. F6/14(4C) x D273-11a gave asci in which a maximum of 2 spores were capable of good growth at 36°C. Several of the remaining spores were difficult to score at 36°C and are marked (?) to indicate this point. Nevertheless, their poor growth at 36°C indicated some

CROSS	TETRAD	SPORE			
		A	B	C	D
F6/14(4 A)	1	+	+	+	+
( <u>MAT</u> ) TR,GR	2	+	+	+	+
x					
F6	3	+	+	+	+
	4	+	+	+	+
F6/14(4 B)	1	+	+	-	+
( <u>MATa,ade2</u> ) TR,gs	2	+	+	+	+
x					
W24	3	+	+	+	+
	4	+	+	+	-
	5	+	+	+	+
	6	+	+	+	+
	7	+	+	+	+
	8	+	+	+	+
	9	+	-	+	+
	10	-	+	+	+
	11	+	-	+	+
	12	+	+	+	+
F6/14(4 C)	1	?	-	?	+
( <u>MATa,met2</u> ) ts,gs	2	-	+	+	?
x					
D273-11a	3	?	+	?	?
	4	+	+	?	-
F6/14(4 D)	1	+	+	+	+
( <u>MAT</u> , <u>ade2</u> ) TR,GR	2	+	+	+	+
x					
F6	3	+	+	+	+
	4	+	+	+	+

TABLE 3.7 SEGREGATION OF TEMPERATURE RESISTANCE

Spores from a single ascus [F6/14(4)] showing a 3:1 segregation for temperature resistance were crossed to appropriate strains. The resulting diploids were sporulated and subject to tetrad analysis. The results presented concern response to the restrictive temperature.

(+) = growth, (-) = no growth, (?) = indeterminable.

manifestation of temperature sensitivity. F6/14(4B) is potentially the most interesting spore. Because F6/14(4A) and F6/14(4D) had been shown to be CDC39, F6/14(4B) was the temperature resistant strain likely to contain a suppressed cdc39 gene. When crossed to strain W24, 7 asci contained only temperature resistant spores, 5 segregated 3:1 TR:ts. Once again a temperature resistant strain had been demonstrated to contain a masked temperature sensitive allele and it was concluded that this strain contained the cdc39 suppressor.

#### Interaction With cdc36

Shusters initial observation was that carbon catabolite derepression could suppress both cdc39 and cdc36. Since both of these are also suppressed by mating type heterozygosity (Connolly, Bugeja, Piggot and Carter, 1983), it had been proposed that they function in a common pathway. Considering this, the effect of the putative suppressor of cdc39 was examined in a cdc36 strain. Mutants 14, 18, 20 and SR665-1 were crossed with SR661-2 (cdc36). The results of the subsequent tetrad analysis are shown in Table 3.8. Unless the cdc39 suppressor was tightly linked to cdc39, and previous data suggested that it was not, the observed results do not fit any model in which an extragenic suppressor is capable of suppressing either cdc39 or cdc36. The control cross, SR665-1 x SR661-2, behaved as expected. Results typical of random segregation indicated that cdc36 and cdc39 were unlinked. The only explanation of the clear 2:2 segregation was that the original mutants 14, 18 and 20 contained revertants of cdc39 with a second mutation conferring resistance to glucosamine.

CROSS	RATIO TEMPERATURE RESISTANCE:TEMPERATURE SENSITIVITY			
	3:1	2:2	1:3	OTHERS
SR665-1 x SR661-2	0	1	10	1
MUTANT 14 x SR661-2	1	27	0	0
MUTANT 18 x SR661-2	0	13	0	1
MUTANT 20 x SR661-2	0	10	0	0

**TABLE 3.8** TETRAD ANALYSIS OF MUTANTS

Strains suspected of carrying extragenic suppressors of cdc39 were crossed with a strain carrying the cdc36 mutation. The resulting diploids were sporulated and subject to tetrad analysis. The segregation of temperature resistance and temperature sensitivity was characterised. The numbers refer to the complete 4-spored asci showing the indicated ratios.



To check this, the mutants were crossed to various strains. When re-crossed to F6 the mutants repeatedly generated temperature sensitive spores, although once again temperature resistance segregated cleanly 2:2 when crossed to SR665-1. If the temperature sensitive alleles did not segregate from the mutants they must be F6 derived. Since F6 grows well at 36°C this would only be possible if these alleles were masked. When F6 was crossed to RC622, a second temperature resistant strain, 3 from 7 asci contained a temperature sensitive spore.

The mutants, including those that had not been investigated were crossed to SMC-18. In this case, the majority of asci from crosses involving 14, 18 and 20 contained no temperature sensitive spores. Indeed, from 48 spores in the SMC-18 x mutant 14 cross, only one showed signs of temperature sensitivity (Table 3.9). The remaining mutants showed much higher frequencies of temperature sensitivity. In each of them the majority of asci segregated 2:2 but a significant number segregated 3:1. This segregation of 3:1 indicated that they may contain suppressors of the cdc39 gene.

#### Complementation Tests

Temperature sensitive segregants from each of the mutant x SMC-18 crosses, were crossed to SR665-1 to check their cdc39 derivation.

The results of this complementation test are shown in Table 3.10. All strains derived from mutants 14 and 18 complemented the cdc39 mutation of SR665-1 and it must be concluded that 14, 18 and 20 contain reversions. None of the other temperature sensitive segregants tested were able to complement cdc39. In

SMC-1B x MUTANT	RATIO TEMPERATURE RESISTANCE:TEMPERATURE SENSITIVITY			
	1:3	2:2	3:1	4:0
6		6	3	
14			1	11
18		1	3	8
20		1	1	10
21		5	3	
22		8	3	
25*				
27		8	3	
28		12		
SR665-1	1	22		

\* This strain segregated 2:2 for growth at 23°C

**TABLE 3.9** TETRAD ANALYSIS OF MUTANTS

Strain SMC-1B was crossed with mutants selected for glucosamine resistance and temperature resistance in a cdc39 background. The resulting diploids were sporulated and the haploid progeny from complete 4 spored asci tested for temperature sensitivity at 36°C. The segregation data are shown. Numbers refer to the number of asci exhibiting the pattern indicated.

STRAIN	SR665-1 x MUTANT			
	HAPLOID		DIPLOID	
	23°C	36°C	23°C	36°C
SR665-1	+	-		
665:2A	+	-	+	-
665:7B	+	-	+	-
6:8B	+	-	+	-
6:1A	+	-	+	-
18:4D	+	-	+	+
18:3C	+	-	+	+
22:8C	+	-	+	-
22:3C	+	-	+	-
21:1D	+	-	+	-
21:4C	+	-	+	-
27:9D	+	-	+	-
27:12C	+	-	+	-
28:7B	+	-	+	-
28:4D	+	-	+	-
F6/14(4 B)	+	+	+	+
F6/14(4 C)	+	-	+	+

TABLE 3.10      COMPLEMENTATION DATA

Temperature sensitive segregants from crosses involving temperature resistant strains carrying putative extragenic suppressors of cdc39 and the WT strain SMC-1B were crossed to SR665-1. Complementation data concerning the response of both haploids and the resultant diploids to temperature is shown.

(+) = growth    (-) = no growth.

all cases the diploid was unable to grow at the restrictive temperature. Failure to complement indicates that mutants 6, 21, 22, 27 and 28 contain the cdc39 gene, and that their continued growth at 36°C is due to suppression and not reversion. As expected, temperature sensitive segregants from crosses involving SR665-1 were unable to complement the cdc39 mutation.

## DISCUSSION

### Carbon Source Effects

The results presented support the observation that the "start" mutants cdc36 and cdc39 are suppressed by carbon catabolite derepression media (Shuster, 1982b). This interaction has important implications for the role of the named genes in cell cycle regulation. Although the details of "start" are unknown, it is clear that this event plays a major regulatory role in coordinating the continuous events of growth and the discontinuous, stage specific events of the division cycle. Catabolite repression is a regulatory mechanism that functions to control carbon metabolism. Since the cell must be aware of the availability of all essential nutrients and be able to respond accordingly, it is not surprising that carbon metabolism and "start" interact.

Suppression of cdc36 and cdc39 indicates that this interaction may proceed via the products of these genes. However, several lines of evidence indicate that this interaction is a complex one and that these genes are not simply involved in informing "start" of the carbon status. Mutations in these genes lead to a G1 arrest in which the cells continue to grow and retain mating competency (Reed, 1980). Any mutation that falsely indicates a nutrient shortage leads to a non-growing, non-mating G1 arrest. Diploids homozygous for either cdc36 or cdc39 fail to arrest in G1 at the restrictive temperature (Shuster, 1982a). Instead, mating type heterozygosity leads to a random arrest. This phenotype is identical to that obtained when cdc36 and cdc39

haploids, growing on pyruvate, are shifted to 38°C.

One could speculate that these data suggest a link between carbon metabolism and start via the mating response. However it is interesting to note that although both derepression and mating type heterozygosity are able to suppress those cdc36 and cdc39 defects relating to regulation at start, they are unable to suppress the growth defect (Reed, 1980, Shuster, 1982a). It has been proposed (Shuster, 1982a) that the products of the cdc36 and cdc39 gene have two functions. One concerned with regulation at "start" in haploids, the other with mass accumulation in all cells. In the absence of evidence it is difficult to state whether the cdc36 and cdc39 gene products have reduced or negligible activity. However, cessation of proliferation at an elevated restrictive temperature implies that these proteins retain a significant activity at 36°C, and that this activity is sufficient for normal growth on derepressive carbon sources. In summary the CDC36 and CDC39 gene products are implicated in response to mating pheromone, carbon metabolism and general growth.

Since the suppression of cdc36 and cdc39 by carbon catabolite derepression has been demonstrated to be dependent only upon the metabolism of a derepressing carbon source, it is clear that some aspect of the biochemistry or morphology of derepressed cells is responsible. Although evidence is scarce, it is possible to speculate what this might be. Several studies (Chapter 1) have indicated that the intracellular cAMP concentration rises in line with the degree of catabolite

repression. Consequently, the concentration of cAMP in cells growing on pyruvate may be significantly higher than in cells metabolising glucose. Although the relationship between cAMP and catabolite repression is a contentious area, a role for cAMP in cell cycle regulation is rapidly gaining support (Chapter 1). CDC28 has been demonstrated to have kinase activity and is itself a substrate for a kinase (Lorincz and Reed, 1983). If the cdc36 and cdc39 gene products are substrates for cAMP dependent protein kinase, mutation of them may lead to a reduction in their affinity for the kinase. In this case, increasing the activity of the kinase by increasing cAMP may overcome the block and provide sufficient levels of cdc gene activity to allow continuing cell cycles. A direct interaction of this type suggests possible allele specificity. Although Shusters evidence concerning cdc28 is weaker than that for cdc36 and cdc39, he does maintain that cdc28-4 is suppressed by carbon catabolite derepression media. This observation has been confirmed by Sudbery (pers. comm.). Table 2 shows that SM6, a strain containing the cdc28-6 mutation, fails to proliferate in derepressive media at the restrictive temperature. Thus, although cdc28-4 is suppressed, the failure of cdc28-6 to continue proliferation indicates an allele specific interaction. This suggestion could be tested by examining several cdc36 alleles, although the single cdc39-1 (Reed, 1984) allele prevents further investigations on this particular gene.

### Isolation of mutants

The clear cut suppression of cdc39 by catabolite derepression media, and the similarity between physiological derepression and the genetic derepression of certain mutants, allowed a strong prediction that suppressors of cdc39 would be found using this protocol (Figure 3.3). Consequently, the isolation of strains acquiring both glucosamine resistance and suppression of cdc39, was expected. Although the mutants selected for further study ultimately proved to be revertants, the steps taken in their characterisation gave several results that were mainly consistent with their description as extragenic suppressors.

1) Figure 3.3 indicates that of the 120 mutants selected for glucosamine resistance, 14 subsequently proved to be temperature resistant. If they were the result of independent mutations. Probability dictated that none of the selected colonies should have had both characteristics. The most reasonable explanation of these data is that both features are the result of a single mutation. Since reversion of cdc39 would not confer resistance to glucosamine, the possibility that the suppressors of cdc39 would be revertants seemed unlikely.

2) Dominance relationships in the 2-15 diploids (Table 3.4) indicated that the suppression of temperature sensitivity was dominant whilst that of glucosamine resistance appeared recessive. Although this is the first evidence that the resistance markers are the result of independent mutations, it says nothing about the nature of either mutation. However, it does raise questions concerning the frequency of appearance of



each mutation.

3) Strain F6 is a CDC39 haploid that grows well at 36°C. When crossed to SR665-1 it gave a clear 2:2 segregation of its temperature resistance (Table 3.5). In this cross its behaviour made it a suitable candidate for studying the segregation of temperature sensitivity. There was no suggestion that this strain contained any temperature sensitive genes.

4) Crosses between the selected mutants and F6 generate temperature sensitive recombinants. The characteristics of this sensitivity and the frequency of its segregation were consistent with it being derived from a suppressed cdc39 strain (Table 3.5).

5) The frequencies of observed phenotypes are in very good agreement with those based on a model following the segregation of three independent genes (Table 3.6). This is further evidence that glucosamine resistance is the result of one gene, and that the suppression of temperature sensitivity is the result of a second gene independent of cdc39.

6) The appearance of an intermediate degree of temperature resistance could be interpreted as partial suppression of cdc39.

7) The segregation frequencies generated during crosses involving SR661-2 (Table 3.8), could only be explained by reversion of the cdc39 gene.

8) When recrossed to F6 the mutants continued to generate temperature sensitive spores. However, a cross in which F6 x RC622 gave temperature sensitive progeny indicated that F6 contains masked temperature sensitive alleles.

9) Failure to complement cdc39 indicated that the temperature

sensitivity of strains derived from mutants 14 and 18, was not due to the cdc39 allele.

These results, presented in chronological order, describe how a series of unfortunate positive results were subsequently uncovered. In retrospect a more cautious approach may have avoided some of these problems. Complementation tests should have been performed on the temperature sensitive progeny from the crosses between F6 and the mutants. However, there was no reason to doubt the derivation of temperature sensitivity and since it was the putative suppressor that was interesting, this aspect of the work was pursued.

Close examination of their response to temperature reveals that the mutants can be placed into three groups. It is now clear that the fast growth mutants have the properties expected of a revertant and that those growing at 36°C but not at 38°C mimic most closely the suppression caused by derepression (Table 3.2). At the time, reversion had been discounted and it seemed sensible to follow the best suppressors of cdc39. With the benefit of hindsight it would have been prudent to study representatives of the three groups.

One of the most misleading indications that the features observed were the consequence of a single mutation, was the frequency at which colonies resistant to both glucosamine and temperature were generated. A speculative explanation may be offered. The selection procedure used employed two distinct selection steps. The first, isolated cells that had acquired glucosamine resistance after EMS mutagenesis. The second step

tested the selected colonies for temperature resistance without further mutagenesis. SR665-1, the parent strain of the mutants had a tendency to spontaneously revert at relatively high frequency. If, during the second selection, the inoculum were sufficiently large, it is possible that the restrictive temperature could force sufficient of these spontaneous revertants, which, given time to incubate, would give a positive score. A possible solution to this would have been to select for glucosamine resistance at the restrictive temperature, but at the time this was considered too stringent.

The results presented clearly indicate that the characteristic of glucosamine resistance is independent of the CDC39 gene. It also appears to be independent of catabolite repression as judged by invertase synthesis. Since glucosamine resistance was simply used as a means of selecting suppressors of cdc39, it was not considered important that the nature of this resistance be determined. It may be that the glucosamine resistance in mutants 14, 18 and 20 is the result of an altered catalytic activity that enables it to metabolise glucosamine.

#### Conclusions

Although the results of the work concerning mutants 14, 18 and 20 have demonstrated them to be simple revertants, the data concerning the other mutants clearly shows that they do contain the cdc39 gene and are not revertants. Several of these mutants give a form of suppression that behaves in many respects like that of catabolite repression. These mutants remain to be characterised but potentially they may be shown to contain

extragenic suppressors of cdc39 that suppress by interaction with the machinery employed in catabolite repression. Although this technique has, as yet, been unsuccessful, it should be used to select suppressors of cdc39. They can subsequently be used to investigate the nature of cdc39 and its interaction with carbon metabolism and mating.

CHAPTER 4

SUPPRESSION BY KNOWN

DEREPRESSION MUTANTS

SUPPRESSION BY KNOWN DEREPRESSION MUTANTSINTRODUCTION

Evidence discussed in chapters 1 and 2, has indicated that carbon metabolism exerts control over aspects of cellular regulation at "start". This evidence takes the form of interactions between carbon catabolite repression, the mating system and certain "start" genes. (Ruiz, Villanueva and Rodrigues, 1984; Shuster 1982a; Connolly, Bugeja, Piggott and Carter, 1983; Shuster, 1982b). The observation that physiological catabolite derepression is capable of suppressing cdc36 and cdc39 (Chapter 2 and Shuster, 1982b) allows the logical prediction that genetic derepression may also be capable of suppressing these genes. Running concurrent with experiments in which extragenic suppressors of cdc39 were isolated (Chapter 3) was a series of experiments designed to test this possibility. I report here the results of experiments in which characterised carbon catabolite repression resistant mutants were crossed into cdc28 and cdc39 backgrounds.

## RESULTS

### cdc28 x grr1

grr1-1 (Bailey and Woodward, 1984) is a recessive mutation characterised by insensitivity to glucose repression of invertase, maltase, galactokinase and cytochrome c oxidase. (Table 1:1, Table 3:6). Associated with this is a change in cell shape such that cell length exceeds cell width by a factor of ten. This gives these cells a characteristic "sausage" phenotype making it easy to follow the segregation of grr1-1. Table 4.1 presents the results of crosses involving strain DGX1-3A (grr1-1). In a cross between cdc28 and grr1-1 strains all asci tested show a 2:2 segregation of temperature resistance to temperature sensitivity. This result was expected since the cross was set up as a control to demonstrate that derepression mutations could not suppress "start" genes insensitive to the "carbon effect". The observation that all progeny from this cross show a parental genotype with respect to the two markers, indicates that linkage between cdc28 and grr1 is tight enough to prevent the generation of recombinants. Although grr1 has not been mapped these data suggest that both cdc28 and grr1 map to chromosome II (Reed, 1980).

### cdc39 x grr1

Seventy five percent of asci in a cdc39 x grr1 cross gave a temperature resistant to temperature sensitive ratio of 2:2. In only 1 of 22 asci tested was a 3:1 ratio observed. On the basis of this it was concluded that grr1-1 was incapable of suppressing cdc39. Assuming random segregation of cdc39 and grr1, and no

CROSS	NUMBER OF ASCI SHOWING TR:ts RATIO			NUMBER OF SPORES SHOWING TR:GR SEGREGATION PATTERNS			
	3:1	2:2	1:3	+/+	+/-	-/+	-/-
TM6 (MAT $\alpha$ , <u>cdc28-6</u> ) x DGX1-3A (MATa, <u>grr1-1</u> )	0	6	0	0	12	12	0
SR665-1 (MAT $\alpha$ , <u>cdc39-1</u> ) x DGX1-3A (MATa, <u>grr1-1</u> )	1	17	4	10*	22	22	10
SR665-1 (MAT $\alpha$ , <u>cdc39-1</u> ) x F6 ( <u>mata</u> , <u>GRR</u> )	2	22	0	50	0	48	0

**TABLE 4.1** SEGREGATION OF TEMPERATURE RESISTANCE AND GLUCOSAMINE RESISTANCE IN CROSSES INVOLVING grr1, cdc28 AND cdc39.

Strains containing the grr1-1 gene were crossed with cdc28-6 and cdc39-1 strains. The resulting diploids were sporulated and the segregation of temperature resistance (TR), temperature sensitivity (ts), and glucosamine resistance (GR) were followed on appropriate media. Results are derived from tetrads in which all 4 spores germinate.

\* Data from 16 asci chosen at random.



selection for any of the observed classes, we would expect to see 25% of each of the classes indicated in Table 4.1. The observed results give a chi squared value of 9 using the above assumptions for the null hypothesis. This chi squared value means that the observed deviation from the expected data, would, under these conditions, occur less than 1 time in 40 by chance alone. Since the experimental design ensures no selection against any class, the deviation must be due to some element of linkage. However, since significant numbers of recombinants were generated it is unlikely that failure to suppress was a consequence of tight linkage in repulsion. Since grr1 appears to be linked to cdc28 (chromosome II) it is difficult to understand linkage between it and cdc39 (chromosome III (Reed, 1984)).

Although the sausage phenotype of grr1 strains acts as a useful visual indicator, Bailey and Woodward (1984) have reported that it is possible to lose this phenotype whilst retaining all other grr1 properties. Considering this, it was felt prudent to score grr1 as sensitivity to glucosamine. A property of grr1 strains not apparent from Table 4.1 was an intermittent growth defect. Of the 50 tetrads derived from a cdc39 x grr1 cross, 25 gave asci in which only 3 spores germinated. In 84% of these cases the missing spore should have been grr1. Although it was not possible to continue work on the fourth spore it was clear that many of them had germinated resulting in micro-colonies.

#### cdc39 x GRR

The SR665-1 x F6 cross was a control. Had DGX1-3A been able to supply a gene capable of suppressing cdc39 this cross would

demonstrate that the supplied gene was not to be found in its parent. The clear 2:2 segregation of both cdc28 and cdc39 genes gives clear indication that these genes behave in a straightforward Mendelian manner.

#### cdc28 x hex2

A second gene exemplifying the major group of derepression genes was isolated by Entian and Zimmermann (1980). This gene, hex2, was crossed into both cdc28 and cdc39 backgrounds. Although only five full asci from the TM6 x SMC-1B/3 cross were examined all gave a clear 2:2 TR:ts segregation (Table 4.2). This is a further illustration that in the absence of suppression, temperature sensitive alleles could be recovered in 2:2 ratios with high frequency.

#### cdc39 x hex2 and CAT1-2<sup>d</sup>

The most interesting cross was that between cdc39 and hex2-3 strains (SR665-1 x SMC-1B/3). Both "stab" and "stringent" tests revealed that approximately 45% of resultant asci contained 3 temperature resistant and 1 temperature sensitive spore (Table 4.2). These results indicated that some feature of SMC-1B/3 is capable of suppressing cdc39, but not the carbon source independent cdc28 gene. SMC-1B/3 contains two mutations related to catabolite repression, CAT1-2<sup>d</sup> and hex2-3. CAT1-2<sup>d</sup> is a mutation that influences the kinetics of derepression without affecting the degree of repression (Zimmermann *et al.*, 1977). To confirm that hex2-3 was the putative suppressor it was necessary to demonstrate that CAT1-2<sup>d</sup> was not suppressing. Although it was possible to separate CAT1-2<sup>d</sup> and hex2-3, the phenotype of the

CROSS		RATIO OF TEMPERATURE RESISTANCE TO TEMPERATURE SENSITIVITY		
		3:1	2:2	1:3
TM6	( <u>cdc28</u> -6)			
	x			
SMC-1B/3	( <u>CAT</u> 1-2 <sup>d</sup> , <u>hex</u> 2-3)	0	5	0
SR665-1	( <u>cdc39</u> -1)			
	x			
SMC-1B/3	( <u>CAT</u> 1-2 <sup>d</sup> , <u>hex</u> 2-3)	9	10	0
SR665-1	( <u>cdc39</u> -1)			
	x			
SMC-1B	( <u>HEX</u> 2, <u>CAT</u> 1-2 <sup>d</sup> )	0	27	0
SR665-1	( <u>cdc39</u> -1)			
	x			
cat2.3-2A/18	( <u>hex</u> 1, <u>cat</u> 2-1)	0	7	0

**TABLE 4.2** SEGREGATION OF TEMPERATURE RESISTANCE AND  
TEMPERATURE SENSITIVITY

The "start" mutants cdc28 and cdc39 were crossed to strains containing mutations affecting aspects of catabolite repression. Diploids were sporulated and the segregation of temperature resistance and temperature sensitivity assayed at 36°C. Figures indicate the number of asci showing the described segregations.

resulting segregant was difficult to identify. Consequently, the effects of this mutation were examined in a background lacking hex2-3 (SMC-1B). Table 4.2 indicates that a cross between a cdc39 and CAT1-2<sup>d</sup> strain resulted in tetrads in which temperature resistance and temperature sensitivity segregated 2:2 in each of the 27 asci examined. Clearly the CAT1-2<sup>d</sup> mutation in isolation is not capable of suppressing cdc39.

Excessive maltose uptake is one of the characteristics of hex2-3 strains. With sufficient maltase activity this results in accumulations of glucose and consequent cell death (Entian, 1980). Using this property, the co-segregation of temperature resistance with the hex2-3 gene was followed on YEP + 3% maltose plates. The results of this are presented in Table 4.3. Asci derived from SMC-1B/3 x SR665-1 that showed the 3:1 segregation of temperature resistance to temperature resistance were examined. As controls, asci showing 2:2 segregations from this cross and asci derived from an SR665-1 x SMC-1B cross were also included. Maltose sensitivity segregated 2:2 and as expected all spores from the SMC-1B control cross were maltose resistant. It should be emphasised that all tests at this stage were of the stringent type. Less demanding "stab" tests continued to give results that were inconsistent. If hex2-3 is capable of suppressing cdc39 it would be predicted that one of the temperature resistant spores contained both a hex2-3 gene and a masked cdc39-1 gene. To identify the hidden cdc39 gene spores showing a temperature resistant, maltose sensitive phenotype were subject to a further round of crosses followed by tetrad

STRAIN	SPORE							
	A		B		C		D	
	TR	MR	TR	MR	TR	MR	TR	MR
SRSMC/5(1)	-	-	+	+	+	-	-	+
SRSMC/5(3)	+	-	+	-	+	+	-	+
SRSMC/5(4)	+	+	-	+	+	-	+	-
SRSMC/6(5)	-	+	+	-	-	+	-	+
SMC-1B/SR665-1(5)	-	+	+	+	+	+	-	+
SMC-1B/SR665-1(3)	+	+	-	+	-	+	+	+

**TABLE 4.3** SEGREGATION OF TEMPERATURE RESISTANCE AND MALTOSE RESISTANCE

SR665-1 was crossed with SMC-1B/3 (hex2-3, CAT1-2<sup>d</sup>) and SMC-1B (CAT1-2<sup>d</sup>).

The resulting diploids were sporulated and the segregation of temperature resistance (TR) and maltose resistance (MR) followed.

(+) indicates resistance.

(-) indicates sensitivity.

analysis.

The crosses carried out and the results obtained are indicated in Table 4.4. This Table indicates several important points. The first is that in all cases temperature sensitive spores can be recovered from temperature resistant parents (see Table 4.5 for controls). Secondly, suppression of cdc39 by hex2-3 precludes the existence of a temperature sensitive, maltose sensitive class of recombinant. These recombinants have been isolated in all Table 4.4 crosses, indicating that the observed suppression is either independent of hex2 or is dependent upon a more complex genetic situation than hex2-3 alone can provide. Thirdly, maltose resistance and maltose sensitivity do not segregate 2:2. In all crosses involving F6, 8 spores were maltose sensitive and 12 maltose resistant. These results are due to the presence of the mal gene from F6. This mutation prevents the cells from metabolising maltose, consequently the accumulation of glucose is limited and the cells remain resistant to maltose. The SRSMC/5(4C) x AM1-9C cross generated 7 maltose sensitive spores and 13 maltose resistant spores. Since there is no mal gene in this cross the reason for the observed segregation of maltose resistance is unclear.

Genetic background may be the explanation for many of the strange phenotypes seen emerging from these crosses. Microscopic examination revealed that approximately 50% of asci generated by SRSMC/5(3A) x F6 and SRSMC/5(4D) x F6 crosses gave a single spore showing an elongated phenotype. Similar aberrant phenotypes were generated by the SRSMC/5(4C) x AM1-9C. At the time these were

CROSS	RATIO OF TEMPERATURE RESISTANCE TO TEMPERATURE SENSITIVITY			SEGREGATION OF TEMPERATURE RESISTANCE AND MALTOSE RESISTANCE TR, MR			
	2:2	3:1	4:0	+ +	+ -	- +	- -
SRSMC/5(3 A) x F6	1	2	2	11	5	1	3
SRSMC/5(3 B) x F6	3	2	0	8	4	4	4
SRSMC/5(4 C) x AM1-9C	5	0	0	9	1	4	6
SRSMC/5(4 D) x F6	0	4	1	10	6	2	2

**TABLE 4.4** SEGREGATION OF TEMPERATURE RESISTANCE AND MALTOSE RESISTANCE

Temperature resistant, maltose resistant haploids derived from a cross between SR665-1 (cdc39) and SMC-1B/3 (CAT1-2<sup>d</sup>, hex2-3) were crossed to either F6 or AM1-9C. The resultant diploids were sporulated and the segregation of temperature resistance and maltose resistance followed. Figures on the left hand of the table refer to the number of complete tetrads showing the described segregations. Those on the right indicate the number of spores from these asci that show the described segregation.

(+) = resistance. (-) = sensitive.

described as being "cdc4 like". No strange cells resulted from the SRSMC/5(3B) x F6 cross and all parents appeared normal. The significance of these results is unknown, as is their genetic basis. They should serve to illustrate that interactions between genes (known and unknown) may lead to complex phenotypes that are difficult to interpret.

cdc39 x hex1 and cat2-1

Table 4.2 indicates that all asci derived from a cross between SR665-1 (cdc39) and cat2.3-2A/18 (hex1, cat2-1) (Zimmermann *et al.*, 1977) contained two temperature sensitive and two temperature resistant spores. This demonstrates that neither hex1 nor cat2-1 are capable of suppressing cdc39.

cdc39 x hxx1 and hxx2

hxx1 and hxx2 (Lobo and Maitra, 1977a) x cdc39 strains were constructed. The resultant haploid progeny gave results that were difficult to interpret. None of the spores from P2T22D x SRSMC/7(7C) cross were temperature resistant at 36°C and the P2T22D 36°C control failed to grow at the restrictive temperature (Table 4.5). Taken together this indicated that P2T22D contained a temperature sensitive allele. Temperature resistance and temperature sensitivity in spores from a P1T8C (hxx2) x SRSMC/6(3B) (cdc39) segregated 3:1. Once again the appearance of 3 temperature sensitive spores suggests problems with the background of P1T8C. These problems could be overcome by crossing and selecting the appropriate recombinants free of the unwanted ts allele.



STRAIN	YEP + 2% GLUCOSE		YEP + 3% MALTOSE	YEPD + 2% GLUCOSAMINE
	23°C	36°C		
TM6	+	-	ND	ND
SMC-1B/3	+	+	-	+
SR665-1	+	-	-	-
SMC-1B	+	+	+	-
cat2.3-2A/18	+	+	-	+
DGX1-3A	+	+	ND	+
F6	+	+	+	-
AM1-9C	+	+	+	ND
SRS MC/5(3:A)	+	+	+	ND
SRS MC/5(3:B)	+	+	+	ND
SRS MC/5(4:C)	+	+	+	ND
SRS MC/5(4:D)	+	+	+	ND
SRS MC/5(3:A) x F6	+	+	+	ND
SRS MC/5(3:B) x F6	+	+	+	ND
SRS MC/5(4:C) x AM1-9C	+	+	+	ND
SRS MC/5(4:D) x F6	+	+	+	ND
P1T8C	+	-	ND	+
P2T22D	+	-	ND	+
SRS MC/7(7:C)	+	-	ND	ND
SRS MC/6(3:B)	+	-	ND	ND
SRS MC/7(7:C) x P2T22D	+	+	ND	ND
SRS MC/6(3:B) x P1T8C	+	+	ND	ND

**TABLE 4.5** SUMMARY OF CONTROLS

Parental strains and other appropriate controls.

YEP = 1% yeast extract + 2% mycological peptone.

YEPD = YEP + 2% glucose.

ND = not done. (+) = growth. (-) = no growth.

### DISCUSSION

Catabolite repression is a cellular manifestation of the need to regulate carbon metabolism. Elements of its diverse nature have been understood for some time (Magasanik, 1961; Polakis and Bartley, 1965; Polakis et al., 1965)) and they indicate that its effects on metabolism are highly pleiotropic. However, more recent work has indicated that catabolite repression also plays a role in regulating the cell cycle. The basic principle upon which the work of this chapter rests is that carbon catabolite repression will suppress cdc36, cdc39 and some alleles of cdc28 (Reed, 1980; Shuster, 1982b). This observation leads to questions concerning the nature of catabolite repression and the mechanism by which carbon metabolism interacts with "start".

The results presented indicate that genes affecting the kinetics of derepression do not suppress cdc39-1. This is not surprising since under the conditions of these tests the cells would remain repressed. The mutations CAT1-2<sup>d</sup> and cat2-1 influence neither the degree of repression nor its relationship with the carbon source (Zimmermann et al., 1977).

grr1 is a mutation that leads to elevated hexokinase activity and insensitivity to glucose repression of several catabolic enzymes (Bailey and Woodward, 1984). Crosses with cdc28 and cdc39 (Table 4.1) indicate neither of these are suppressed by grr1-1. Although this mutant was isolated by resistance to 2-deoxyglucose, mutations of this type also confer cross resistance to D-glucosamine (Lobo and Maitra, 1977a). Consequently, glucosamine was used to follow the segregation of

this and other genes isolated by resistance to 2-deoxyglucose.

hex1 leads to a 30% reduction in hexokinase activity. Biochemical evidence has demonstrated that these mutants lack hexokinase P2 (Entian and Mecke, 1982). Once again a mutation, capable of inducing the constitutive derepression of certain cytoplasmic and mitochondrial enzymes (Entian and Zimmermann, 1980), failed to suppress cdc39-1 (Table 4.2).

Although Table 4.2 indicates that hex2-3 suppresses cdc39, suppression in only 50% of the asci tested is an observation that needs to be explained. Table 4.4 reveals the presence of maltose sensitive spores that are also temperature resistant. Their existence renders a model that predicts cdc39 will be suppressed by hex2-3 independent of any other gene invalid. The presence of masked temperature sensitive alleles in F6 (demonstrated in Chapter 3) complicates the interpretation of the data in Table 4.4 concerning the segregation of temperature resistance. Nevertheless, data regarding the SRSMC/5(4C) x AM1-9C cross indicate that temperature sensitive alleles can be recovered as expected. One would therefore expect that in the absence of suppression more temperature sensitive alleles rather than less would be seen.

A speculative explanation for these results may be that suppression of cdc39 by hex2-3 is dependent upon the presence of a second gene. Evidence that the expression of aspects of the hex2-3 phenotype may be dependent upon other genes comes from several quarters. Although maltose sensitivity is a relatively easy marker to score, the crosses of Table 4.4 consistently show

an excess of spores with a maltose resistant phenotype. The reason for this is that sensitivity to maltose is only expressed in combination with genes that allow maltose metabolism. Since strain F6 contains the mal gene, spores inheriting both hex2 and mal will be phenotypically maltose resistant. Although grr1 and hex2 are not allelic, they show many phenotypic similarities. Indeed, it was these similarities that prompted Bailey and Woodward (1984) to examine allelism. Both mutants show increased hexokinase activity. In the hex2-3 mutant this has been attributed to increased production of hexokinase isoenzyme P2 (Entian, 1981). In neither case is the primary defect known. Entian and Zimmermann (1980) concluded that "hex2-3 mutants appeared to be somewhat affected in the regulation of synthesis of the components of this system (i.e. hexose phosphorylation system)". Since grr1 is not allelic to hex1/hxk2, the structural gene for hexokinase P2 (Lobo and Maitra, 1977b), and since only mutations in hexokinase P2 are correlated with catabolite repression (Entian and Mecke, 1982; Entian and Frohlich 1984), it too is probably a regulatory gene. The failure of Lobo and Maitra to isolate a strain that had, as the result of a single mutation, simultaneously lost hexokinase P1 and P2 activities indicates that they are independently regulated (Lobo and Maitra, 1977b). Consequently, both hex2-3 and grr1-1 are probably part of the same P2 regulatory mechanism.

The important point about this comparison is not their undoubted similarity but the nature of their differences. Since the grr1-1 strain is not maltose sensitive, even in the presence

of maltose utilisation genes, it is clear that maltose sensitivity is not simply a consequence of enhanced hexokinase activity. The previous assumptions also predict that the catabolite repression resistant properties of the hex2-3 strain may be independent of maltose sensitivity. This situation may be similar to that of the grr1 "sausage" phenotype. Initially this phenotype was thought to be tightly linked to the 2-deoxyglucose resistance of grr1, however, this association was lost in later crosses. Conclusive evidence for this argument may have been gained had hexokinase activity and 2-deoxyglucose resistance been followed in the crosses of Table 4.4.

The argument as presented, has concentrated on discussing the possibility that, as with maltose sensitivity, the expression of other aspects of hex2-3 are influenced by interactions with other genes. Putative interactions could explain why only 50% of asci from the SR665-1 x SMC-1B/3 cross show evidence of suppression. It is possible that some aspect of the hex2 phenotype interacts with a second gene to effect suppression of cdc39. In this scheme a maximum of 50% of asci would generate a spore in which the cdc39 gene is in combination with both hex2 and the second gene. Absence of the second gene could explain why grr1, phenotypically similar to hex2, does not suppress cdc39. If the failure of grr1 were due to the absence of a second gene, both SR665-1 and DGX1-3A must lack the appropriate allele of this gene. If this is the case the gene cannot be involved in the suppression caused by derepressive media. The implication being that physiological and genetic suppression of

cdc39 are not identical.

Although I have shown that neither grr1-1 nor hex2-3 are capable of suppressing cdc28-6 (Tables 4.1 and 4.2). This does not exclude the suppression of other alleles of cdc28 in the manner demonstrated by derepressive carbon sources (Chapter 3).

It is unknown whether or not cAMP has a role in suppression by hex2. Since cAMP measurements have not been performed on these mutants, Entian and his colleagues presumably believe that this nucleotide is not involved. Michels, Hahnenberger and Sylvestre (1983) have isolated, by resistance to glucosamine, a mutant, glr1, that is allelic to hex1 and hvk2. Although they have not measured cAMP, preliminary assays have found that the levels of adenylate cyclase and cAMP phosphodiesterase show no significant differences from the GLR1 parent. This does not preclude the possibility that cAMP levels are affected in the suppressing hex2-3 mutant, but the absence of any positive data makes this unlikely.

#### CONCLUSIONS

The evidence suggests that some element of derepression is capable of suppressing cdc39. However, the nature of the suppression remains unknown. It is also difficult to identify the reasons behind the difference in behaviour shown by hex2-3 and grr1-1. Having postulated that the reason may be the absence of a missing gene, it is possible, even though the two genes may function on the same pathway, that suppression is due to a characteristic of hex2-3 that grr1-1 lacks. If a second gene product, acting in concert with, or as the result of modification

by, some aspect of the hex2-3 pleiotropy is responsible for suppressing cdc39, this gene may be a candidate for the extragenic suppressors of cdc39 isolated in Chapter 3.

CHAPTER 5

cAMP ASSAYS



## cAMP ASSAYS

### INTRODUCTION

We have proposed a model in which cAMP plays an important role in cell cycle regulation (Figure 1.3). Evidence for this model is mainly circumstantial and is based upon the aberrant cell cycles of mutants defective in cAMP metabolism (Chapter 1; Matsumoto, Uno and Ishikawa, 1985a; Jacquet and Camonis, 1985). These mutants (bcy1) (Matsumoto et al., 1982a) illustrate that enhanced phosphorylation by cAMP dependent protein kinase leads to continued growth under conditions in which the wild-type would arrest. The absence of cAMP dependent phosphorylation (cyr1. Matsumoto et al., 1982a) prevents growth where the wild-type would proliferate. They indicate the extremes of phenotype that are dependent upon indirect regulation by cAMP and that the effects of cAMP in the wild-type operate solely via cAMP dependent protein kinase. Although the mutants demonstrate the results of maximal and minimal effective concentrations of cAMP, the relationship between the absolute or relative concentrations of cAMP, protein kinase activity and the resultant phenotype are unknown.

Since the phenotypes of enhanced and decreased phosphorylation mutants are analogous to exponential growth and nutrient starved G1 arrest, these wild-type phenotypes may be accompanied by high and low cAMP respectively. By assaying cAMP it was hoped that fluctuations consistent with these predictions could be demonstrated.

### RESULTS

The direct measurement of cAMP is probably the most effective method of providing evidence relating to any model based on cAMP fluctuations. For meaningful measurements of cAMP certain conditions must be met to ensure reproducibility. Cellular fluctuations of cAMP can be very rapid (van der Plaats and van Solingen, 1974). To minimise these fluctuations, cells must be fixed as soon as possible after sampling. The method of preparing crude cell extracts must consistently liberate maximal amounts of cAMP and care must be taken to ensure that contaminants interfering with the assay, or enzymes that produce or destroy cAMP are removed.

To ensure that maximal liberation of cAMP was achieved, a series of experiments using labelled histidine as an indicator molecule were carried out. The format of these experiments was to preload exponentially growing cells with labelled histidine, assay the amount taken up and, by adopting several liberation protocols, determine the method giving maximal, reproducible recovery. Histidine was chosen as the indicator because of its comparable size to cAMP. Labelled cAMP could not be used because low permeability limited the accumulation of sufficient amounts of label. It was assumed that after a ten minute preloading, the majority of labelled histidine would still be free and cytoplasmically located.

Method one involves treating the cells with a combination of toluene, a permeabilising agent, and sonication. From the results of this experiment several conclusions can be made.

A) Table 5.2 shows that increasing concentrations of toluene increases the liberation of histidine and that the histidine is water soluble.

B) Figure 5.1 depicts the relationship between length of sonication and the release of label and indicates that large amounts of histidine were freed by long periods of sonication.

C) Tube 8 (29%) indicates that the effects of toluene and sonication are synergistic. This value exceeded the additive total given by both 1 minute sonication (tube 9 (19%)) and 25% toluene (tube 6 (3.3%)).

It was concluded that sonication was particularly effective at permeabilising cells. However, long periods of sonication were necessary to effect maximal release and this was inconsistent with the need for rapid assaying. Consequently, a combination of moderate lengths of sonication with toluene treatment seemed the best approach.

The next question to be addressed was the length of toluene incubation needed for effective permeabilisation. Figure 5.2 shows how effective the combination of sonication and toluene treatment was and that 10 minutes incubation with 25% toluene was sufficient to produce its maximum permeabilisation. The plateau after 10 minutes was an indication that this method consistently liberates maximal levels of histidine.

One of the criteria used in deciding on a method for releasing cAMP was that it should not interfere with the subsequent cAMP assay. An experiment was performed to measure possible interference of sonication or toluene with the binding

TUBE	CONTENTS	REAGENT 1 (BUFFER)	REAGENT 2 (BINDING PROTEIN)	REAGENT 3 [8- <sup>3</sup> H]cAMP	STANDARDS	UNKNOWNNS
1 & 2	Charcoal blank	150	--	50	--	--
3 & 4	Zero dose	50	100	50	--	--
5 & 6	1 pmol standard	--	100	50	50	--
7 & 8	2 pmol standard	--	100	50	50	--
9 & 10	4 pmol standard	--	100	50	50	--
11 & 12	8 pmol standard	--	100	50	50	--
13 & 14	16 pmol standard	--	100	50	50	--
15 etc.	Unknowns	--	100	50	--	50

TABLE 5.1 CONTENTS OF cAMP ASSAY TUBES

The contents of tubes in the cAMP assay. The figures refer to  $\mu$ l contents of each of the tubes. Reagent 1 = tris/EDTA buffer pH 7.5 (0.05M solution containing 4mM EDTA). This table is reproduced from the Amersham cAMP assay kit, product information handbook.

FIGURE 5.1      RELEASE OF LABELLED HISTIDINE BY SONICATION

Following preloading with labelled histidine (as described in materials and methods) cells were subject to increasing periods of sonication. After centrifugation the amount of label was assayed. Effectiveness (% release) is expressed as (cpm of intact cells / cpm of the supernatant following treatment) x100

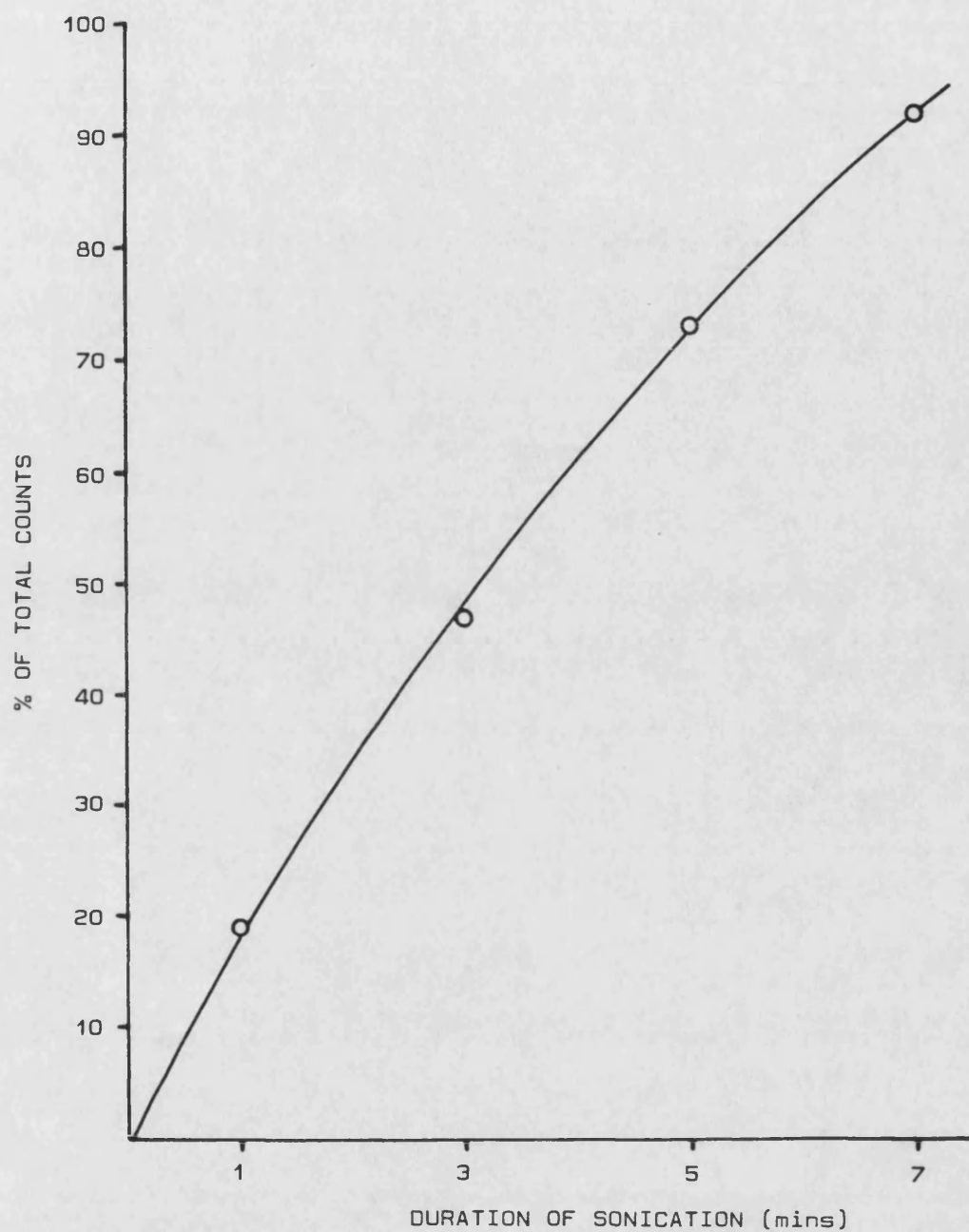
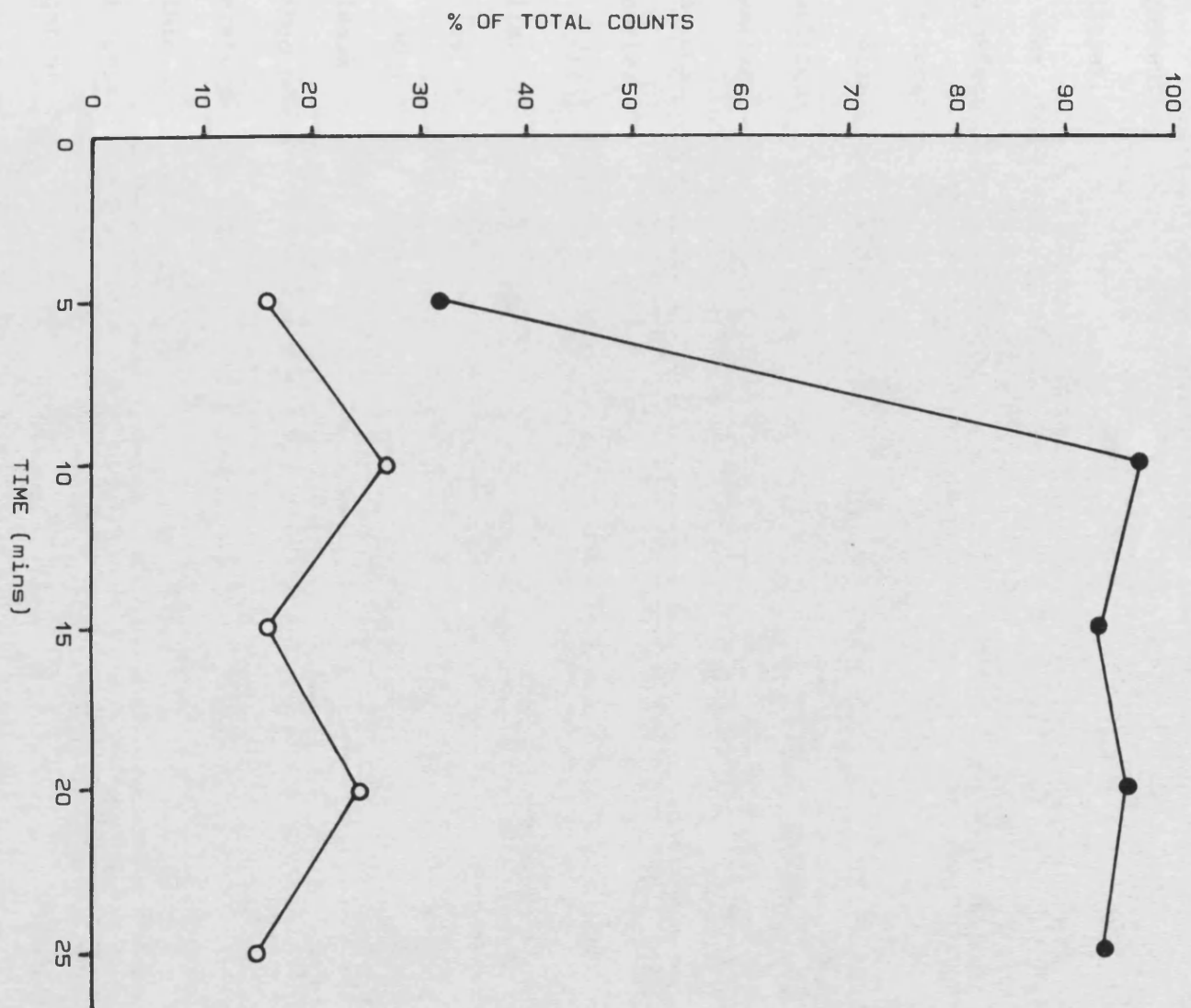


FIGURE 5.2      RELEASE OF LABELLED HISTIDINE FOLLOWING TREATMENT  
WITH SONICATION OR TOLUENE

The effectiveness of toluene in promoting the release of labelled histidine was examined. Following preloading with histidine (as described in materials and methods) cells were incubated for increasing periods in the presence of 25% toluene with ( ● ), or without ( ○ ) 3 minutes sonication. Effectiveness (% release) is expressed as in Figure 5.1.





protein assay (Figure 5.3). This suggested that although neither treatment alone could affect the results of a standard calibration curve, used together the results could be distorted. It may be that sonication had an effect on toluene such that it now affected the binding protein. On the basis of this result this treatment was abandoned and a second method sought.

Attempts were made to open cells using a glass bead fractionation method. This method, used in the assay of invertase, liberated a maximum of 60% total label. This yield was considered unacceptable since the concentrations of cAMP were expected to be low.

Freeze/thawing is a well documented method of breaking cells. Its effectiveness is illustrated by Figure 5.4. Three cycles of freeze/thawing were sufficient to give maximum recovery of label. Once again the plateau indicated the consistency of release that was essential to ensure reproducible results. This method has several advantages, cAMP is cold stable and is therefore unlikely to be affected by it and rapid freezing will, within a few seconds, lead to an equally rapid inactivation of all enzymes including those involved in cAMP metabolism. In light of this, freeze/thawing was adopted as the best method for disrupting cells.

#### cAMP Measurements

Rapid fixing of cell samples was achieved by adding trichloroacetic acid (TCA). This reagent permeabilises the cells and starts the liberation of cAMP which is continued by freeze thawing. Table 5.3 contains the results of an experiment testing

FIGURE 5.3      EFFECT OF TOLUENE AND SONICATION ON THE cAMP ASSAY

Possible interference of toluene and/or sonication on the cAMP assay was examined. A series of standard curves were constructed and subject to the following treatments prior to cAMP determinations. Lines plotted as in Figure 2.2

( ■ ) standard calibration curve - no treatment.

( □ ) 25% toluene.

( ● ) 3 minutes sonication.

( ○ ) 25% toluene + 3 minutes sonication.

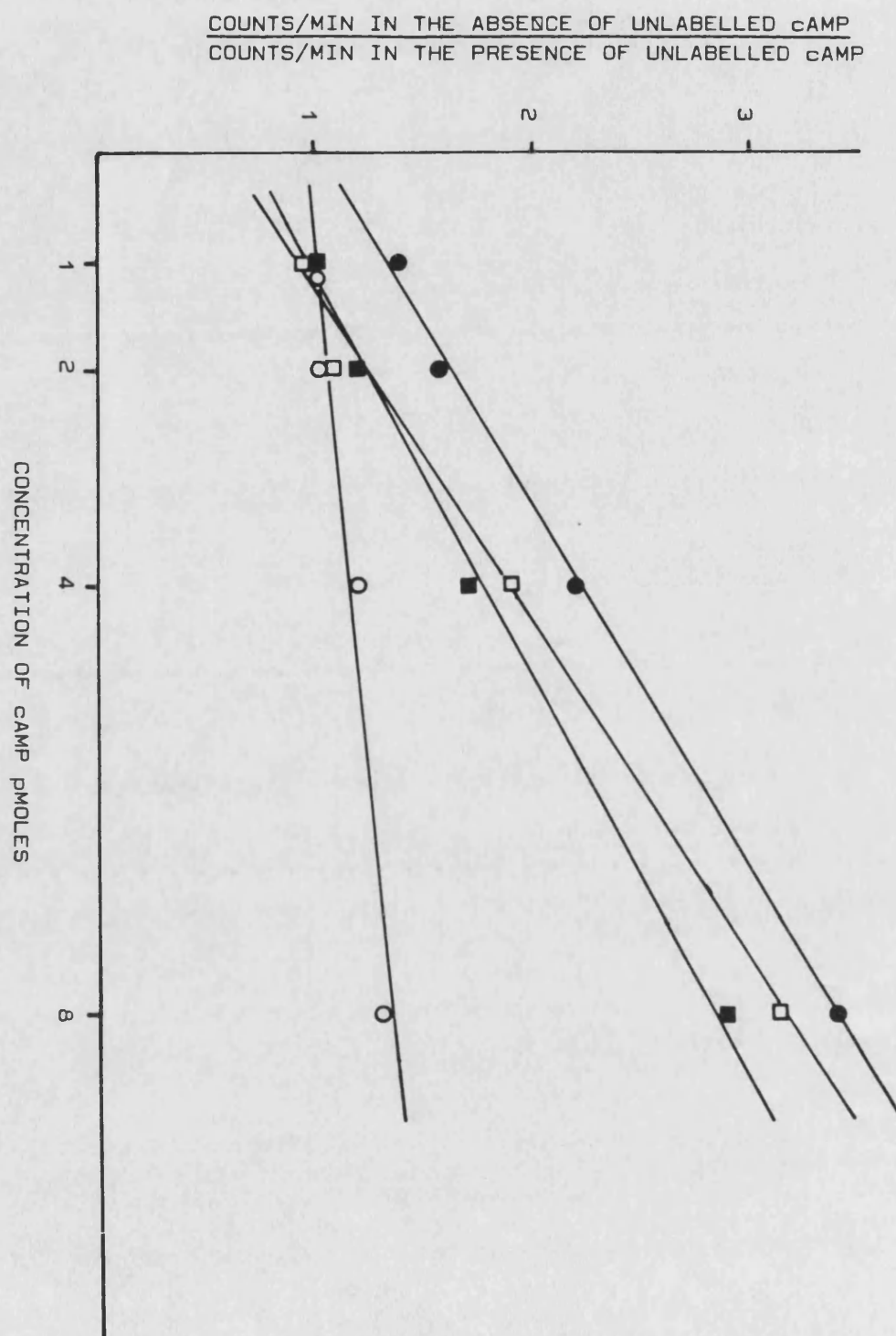
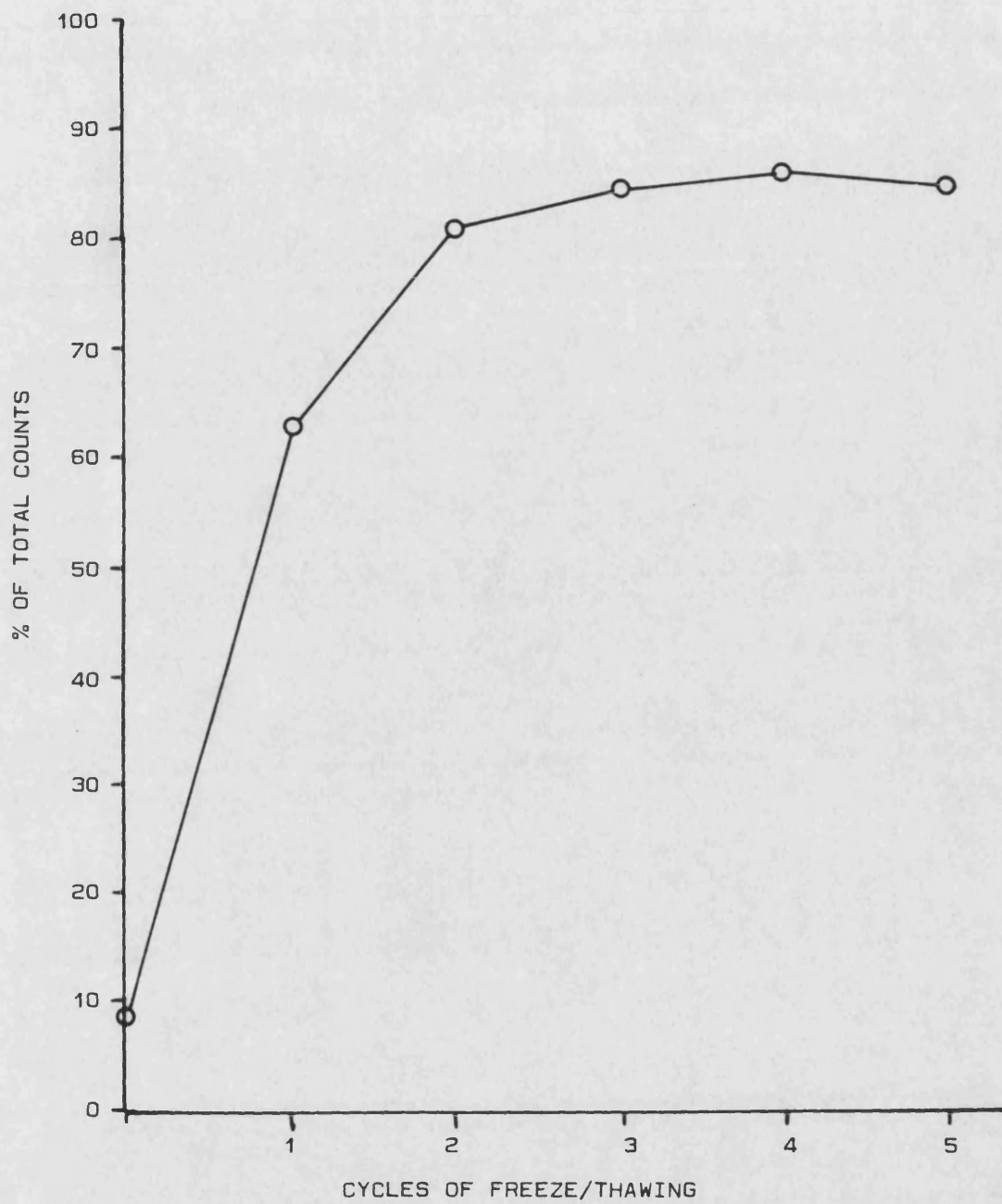


FIGURE 5.4      RELEASE OF LABELLED HISTIDINE BY FREEZE/THAWING

Cells were preloaded with labelled histidine (as described in materials and methods) and then subject to cycles of freeze/thawing. The release of labelled histidine into the supernatant was assayed in a scintillation counter. Effectiveness (% release) is expressed as in Figure 5.1



<u>TUBE</u>	<u>SAMPLE</u>	<u>CPM</u>	<u>[cAMP]pmol</u>
1	Zero dose	6496	
2	1 pmol standard	4131	1.51
3	2 pmol standard	3417	2.38
4	4 pmol standard	2569	4.05
5	8 pmol standard	1683	7.60
6	Sample 1 (no extraction)	12580	
7	Sample 2 (2 extractions)	4045	1.61
8	Sample 3 (4 extractions)	4011	1.64
9	Sample 4	2678	3.71
10	Sample 5	2520	4.24
11	25µl sample 4 + 25µl 8pmol standard	1289	10.70
12	25µl sample 4 + 25µl 4 pmol standard	1056	13.64
13	25µl sample 5 + 25µl 8 pmol standard	1151	12.30
14	25µl sample 5+ 25µl 4 pmol standard	1155	12.2
15	50µl labelled cAMP (Total counts)	48254	

TABLE 5.3      cAMP ASSAY RESULTS

The results of a cAMP binding protein based assay on various samples and controls. Tubes 1 to 5 are cAMP standards. Tubes 6 to 10 are samples isolated from cells of the same flask treated in different ways. Tubes 11 to 14 are control tubes. Tube 15 indicates the total counts obtained from the 50µl of labelled cAMP added as part of the assay. The results presented are the average of two identical tubes.

for interference from TCA on the cAMP assay. Tube 6 is a sample lacking the ether extraction of TCA. This tube gave a very high reading and it was likely that TCA interfered with the assay. Tube 7 (2 extractions) gave a reading similar to that of tube 8 (4 extractions) indicating that TCA contamination was removed by two extractions with ether.

Tubes 11-14 were control tubes in which a known concentration of cAMP was added to samples. The reasoning for this is that it should be possible to predict minimum assayed values of cAMP. The results obtained were not expected since they show no consistency. Since tubes 6 to 10 were samples prepared from the same cell culture, tubes 7 to 10 should have given similar cAMP concentrations. The control tubes indicated that the assay seemed to give cAMP concentrations that were independent of the actual concentration. These data suggested that some component of the cell free supernatant was capable of interfering with the assay, giving spurious results.

A second attempt to measure cAMP was completed on cells growing on 5% glucose and 1.5% pyruvate, with cells harvested at  $6 \times 10^6$  cells/ml and  $9 \times 10^6$  cells/ml respectively. Under these conditions cells using pyruvate could be expected to have higher levels of cAMP (Mahler and Lin, 1979; Van Wijk and Konijn, 1971).

Predictions for this experiment were that more cAMP would be isolated from cells growing on pyruvate and that twice the absolute amount of cAMP would be isolated from 60 mls of a culture than 30 mls of the same culture. It was felt that the problems seen in previous assays may have been the result of very

low concentrations of cAMP. With this in mind, large numbers of cells were used for the assay and cAMP was further concentrated by freeze drying. The results of this experiment are presented in Table 5.4. Once again the results were inconsistent. In general the agreement between identical samples was very poor. In the case of the 30 ml glucose cultures this difference was almost an order of magnitude. The between samples agreement was also very poor, 30 mls of pyruvate grown cells seeming to contain more cAMP than 60 mls of the same culture. The only "expected" result is the observation that pyruvate grown cells contain more cAMP than glucose grown cells. However, considering the previous results this could have been due to chance.

The freeze dried results are difficult to explain. The counts per minute were very much higher than the zero dose control that indicated the maximum amount of label bound in the absence of cAMP. Tube 15 of Table 5.3 indicated that, as part of the cAMP assay, the 50  $\mu$ l of labelled cAMP added gave a total cpm of 48254. The zero dose control for this experiment (tube 1 Table 5.3) gave a cpm of only 6496 and suggested that the binding protein binds a small (14%) percentage of the total labelled cAMP. From this it was clear that the freeze dried results can be explained in two ways. It was possible that concentrating that component of the cell supernatant that was supposedly responsible for interference with the Amersham assay led to increased binding of the labelled cAMP. A second and more plausible explanation was that the putative interfering component interacted with either cAMP or the charcoal adsorbent preventing



TABLE 5.4      cAMP ASSAY RESULTS

cAMP levels of cells grown on repressive or derepressive carbon sources. The cells were grown to mid-exponential phase in YEP + 3% sodium pyruvate or YEP + 5% glucose. cAMP was isolated and assayed as described in "materials and methods". The results presented are the average of two identical tubes. Freeze dried samples were initially assayed as described for other samples, 0.8 mls was then freeze dried and resuspended in 110  $\mu$ l of 0.05M tris EDTA buffer (pH 7.5).

SAMPLE	AVERAGE CPM-BLANK	Co/Cx	[cAMP] pmol	[cAMP] pmol/10 <sup>8</sup> cells
Zero	7044	1		
1 pmol standard	3251	2.16	1.102	
4 pmol standard	1479	4.76	3.57	
16pmol standard	412	17	15.2	
60ml Glucose (1)	601	11.7	10.1	2.85
60ml Glucose (2)	834	8.4	7.03	1.98
30ml Glucose (1)	3400	2.07	1.016	0.57
30ml Glucose (2)	6149	1.14	0.133	0.075
60ml Pyruvate (1)	801	8.79	7.4	1.37
60ml Pyruvate (2)	382	18.4	16.5	3.05
30ml Pyruvate (1)	373	18.9	17.0	6.3
30ml Pyruvate (2)	282	24.9	23.9	8.8

#### FREEZE DRIED SAMPLES

SAMPLE	COUNTS PER MINUTE	
	REPLICATE 1	REPLICATE 2
60ml Glucose	19169	20289
30ml Glucose	21271	14340
60ml Pyruvate	19049	13093
30ml Pyruvate	9186	11535

TABLE 5.4      cAMP ASSAY RESULTS

the precipitation of cAMP. The result of this would be an increase in the concentration of labelled cAMP in the supernatant with a consequential increase in the counts per minute.

This work forced me to conclude that the assay kit as used could not give accurate or reproducible results and that the measurement of cAMP should wait until more sensitive and reliable methods were available.

### DISCUSSION

The ubiquitous nature of cAMP and its demonstrated regulatory roles in both prokaryotes and eukaryotes, have generated a great deal of interest in this nucleotide. In the course of its study several cAMP assays have been developed. Indeed cAMP, its metabolism and its assays are a frequently reviewed subject (Goldberg and O'Toole, 1971; Jost and Rickenberg, 1971; Brooker, 1975). Early assays exploited the chemotactic response of Dictyostelium discoideum (Van Wijk and Konijn, 1971). More recently, assays based on a cAMP binding protein (Sy and Richter, 1972a; Hixson and Krebs, 1980) have been developed (Gilman 1970, Brown et al., 1971).

Although these assays have been used in yeast, the results obtained show wide fluctuations. Table 5.5 contains a summary of the results of some of these assays. The figures quoted are in the form presented by each of the authors. To allow a more direct comparison to be made conversion based on the following assumptions can be carried out: A)  $2 \times 10^7$  cells = 1 mg/protein; B) 1 pmol/mg/protein =  $0.027 \mu\text{M}$  (Mahler and Lin, 1979). Using these conversions the reported concentration range is between  $0.027 \mu\text{M}$  and  $10 \mu\text{M}$ . This represents a 370 fold change in the reported levels of cAMP. The highest level reported by Matsumoto et al. (1985a) is seen only in a mutant containing mutations that result in both increased adenylate cyclase activity (IAC) and reduced phosphodiesterase activity (pde1) (Uno et al., 1983a). An explanation for the wide variations exemplified by Table 5.5 may be the difficulty of reproducible cAMP assays. These

REFERENCE	EXTRACTION & cAMP ASSAY	[cAMP]
Eraso & Gancedo, 1984	Filtration and TCA extraction Centrifugation and TCA extraction Centrifugation and acetic acid extraction cAMP assayed using Amersham kit	0.04 - 10 $\mu$ M
Mahler & Lin, 1979	Boehringer cAMP assay kit	0.35 - 0.85 $\mu$ M
Schlanderer & Dellweg, 1974	Acetic acid extraction & freeze/thawing cAMP assay as in Gilman, 1970	12 - 472 pmol/gm dry wt
Tortora <u>et al.</u> , 1982	Filtration, acetic acid extraction cAMP assayed using Boehringer kit	0.5 - 5.0 $\mu$ M
Matsumoto <u>et al.</u> , 1985a	Filtration, homogenisation & TCA extraction cAMP assayed as in Brown <u>et al.</u> , (1971) and using the Amersham kit	1 - 20 pmol/mg/protein
Van der Platt & van Solingen, 1974	Perchloric acid extraction & freeze/thawing Assayed using BDH binding protein based on Brown <u>et al.</u> , 1971	1 - 10 $\mu$ M
Watson & Berry, 1977a & 1977	TCA extraction, assayed using Boehringer kit	13 - 52 pmol/5x10 <sup>7</sup> cells 5 - 45 pmol/5x10 <sup>7</sup> cells
Wijk & Konijn, 1971	Perchloric acid extraction & freeze/thawing cAMP assayed using <u>Dictyostelium discoideum</u>	0.04 - 0.32 $\mu$ M

**TABLE 5.5**      A SUMMARY OF cAMP ASSAY PROCEDURES  
A summary of the methods used and the results obtained in the  
assay of cAMP from Saccharomyces cerevisiae.

difficulties have been reported by several laboratories, each claiming that the assays presently used are not sufficiently sensitive, accurate or reproducible (Sudbery, P., pers. comm.; Dickinson, J. R., pers. comm.; Liao and Thorner, 1980; Thorner, J., pers. comm.).

Although the sampling of cAMP is very important in the reproducibility of the assay, it is a point rarely mentioned in the literature. Nevertheless, the most frequently used methods are very similar to those that I employed. Most procedures include an acetic acid, perchloric acid or trichloroacetic acid extraction, followed by freeze/thawing. Eraso & Gancedo (1984) have conducted a systematic investigation of cAMP assays in an attempt to resolve the large discrepancies in reported cAMP levels between groups using similar methods. Their approach demonstrated that the choice of isolation procedure failed to make a significant difference to the measured cAMP level (Table 5.5). Their results, contrary to the data of many researchers, state that cAMP levels are higher in repressed cells than in derepressed cells. Although the absolute relationship between cAMP and catabolite repression is important, the most valuable lesson given by their work is that cAMP assays frequently give results that they describe as "artefacts" or "erratic". Having demonstrated that sampling procedures are not critical, they conclude that discrepancies in the literature are "due to the differences in the determination of cAMP itself". Since all their cAMP assays were carried out using the Amersham kit, they were unable to provide evidence to support this claim. Clearly

the sensitivity and accuracy of the selected cAMP assay will be crucial in the reproducible assesment of cAMP levels. There are several points in the results pertaining to these features. The Amersham kit is claimed to be most sensitive in the 0.5 to 4 pmol range. Figure 5.3 indicates that as cAMP levels fall to 1 pmol the Co/Cx ratio approaches 1. At this point it becomes impossible to assay cAMP. The cAMP standards of Tables 5.3 and 5.4 show significant deviations from their expected concentrations. Although it is easy to explain these discrepancies in terms of inaccuracy, all these determinations were carried out with great care. If these errors are due to sampling or pipetting inaccuracies they are difficult to avoid.

Although changes in the intracellular cAMP level were sought in support of our proposed model, it is clear that these changes would only be necessary to effect changes in the activity of cAMP dependent protein kinase. Since stimulation of protein kinase is dependent upon the binding of cAMP, it is the level of bound cAMP rather than free cAMP that is actually important. The cAMP assays that have been described measure the amount of free cAMP. Before changes in the level of free cAMP can be associated with regulation of the cell cycle, it will be necessary to understand the relationship between the levels of free and bound cAMP.

Some indication of the range of cAMP concentrations necessary to cause maximal and minimal regulation of protein kinase can be obtained from the data of Matsumoto et al. (1985a).

The cyr1-2 mutation reduces the activity of adenylate cyclase leading to intracellular cAMP levels of 0.4 pmol/mg/protein.

This reduction in cAMP is sufficient to result in the phenotype given by cells in which protein kinase activity is negligible (cyr2). Clearly the levels of intracellular cAMP would have to be higher than 0.4 pmol/mg/protein to give significant protein kinase activity. The pde1 mutation gives intracellular cAMP levels of 6.7 pmol/mg/protein. The phenotype of this mutant is identical to that of a mutation in which the catalytic subunit of cAMP dependent protein kinase is free to operate with maximal activity (bcy1). It is concluded that this level of cAMP is sufficient to induce maximum cAMP dependent protein kinase activity. From these mutants it is clear that fluctuations in levels of cAMP between 0.4 and 6.7 pmol/mg/protein are more than sufficient to effect the changes in protein kinase activity seen in wild-type yeast. The actual effective concentration range may prove much narrower than this. If this is the case it may prove very difficult to measure the changes in cAMP levels, possibly subtle, that may occur throughout the cell cycle. To further confuse matters, if cAMP acts as a regulatory molecule, the mechanism used must be able to differentiate between changes in cAMP designed to affect cell cycle regulation from those occurring in response to a changed catabolite repression status.

#### CONCLUSIONS

The work of this chapter has demonstrated that sonication, freeze-thawing and toluene permeabilisation, are all effective methods of freeing intracellular label. However, problems with interference and local increases in temperature make cycles of freeze-thawing the method of choice.



Although the cAMP metabolism mutants have demonstrated a link between cAMP and cell cycle regulation at "start", the nature of this link remains to be resolved. What is clear is that in my hands and the hands of several others, the cAMP assay cannot successfully be used to assay cAMP in Saccharomyces cerevisiae. The questions concerning the role of cAMP in the regulation of the cell cycle must wait until more sensitive cAMP assays have been developed.

CHAPTER 6

EFFECT OF IBMX;  
STARVATION OF bcy1;  
INTERACTION OF ste  
MUTANTS WITH cdc39.

## INHIBITION OF cAMP PHOSPHODIESTERASE

### INTRODUCTION

Isobutylmethylxanthine (IBMX), an inhibitor of cAMP phosphodiesterase, has been used to overcome alpha-factor arrest (Liao and Thorner, 1981). The effect of this compound is to increase the intracellular cAMP levels by preventing its degradation to 5'AMP (Aboud and Burger, 1971; Liao and Thorner, 1981). Carbon catabolite derepression media are capable of suppressing cdc36 and cdc39 at 36°C (Shuster, 1982b), and although there are conflicting results, evidence suggests a link between the degree of repression and the intracellular cAMP concentration (Chapter 1). Consequently, suppression of cdc36 and cdc39 may be the result of either a rise in the concentration of cAMP or one of the other pleiotropic effects of derepression (Polakis and Bartley, 1965; Polakis et al., 1965). Distinguishing evidence may be obtained by suppressing cdc39 using a compound that affects the cAMP levels, without affecting the repression status. Tsuboi and Yanagishima (1973) used methylxanthines to demonstrate that increases in both cAMP levels and the degree of sporulation, occur as a result of inhibition of phosphodiesterase. In this way it was hoped to demonstrate that IBMX could suppress cdc39 in a manner analogous to its effects on mating (Liao and Thorner, 1981) and sporulation (Tsuboi and Yanagishima, 1973).

### RESULTS AND DISCUSSION

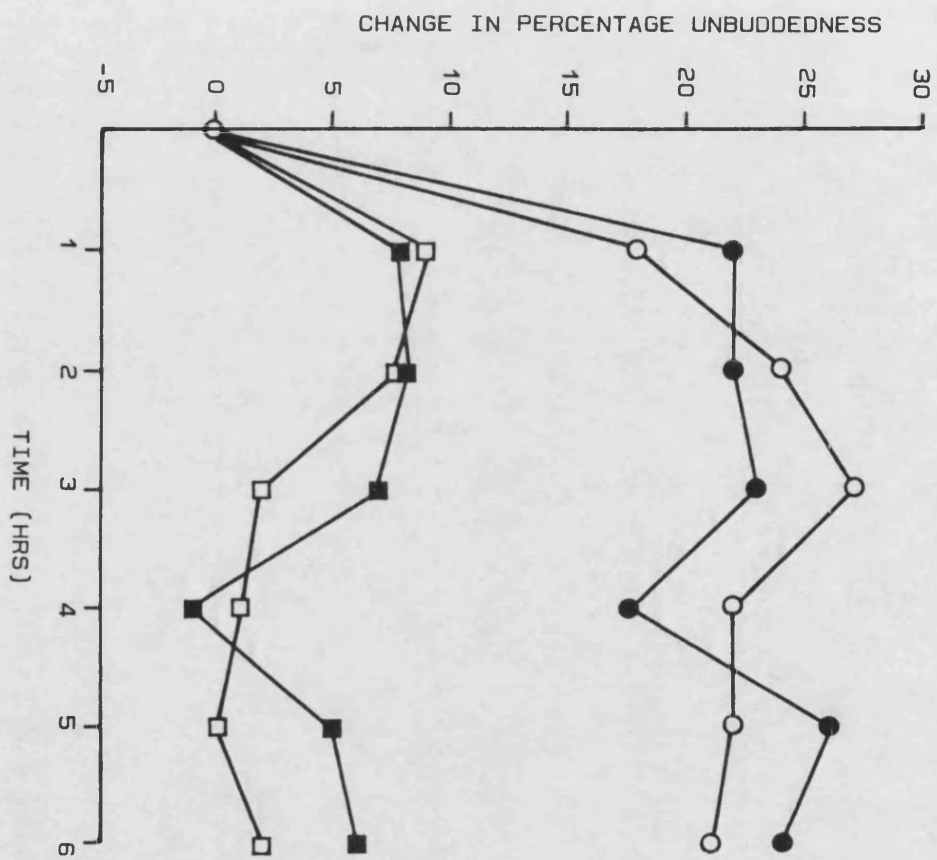
The results of these experiments were generally disappointing. Although several attempts were made, it proved impossible to

repeat Liao and Thorner's (1981) results with alpha-factor. Nevertheless, the effects of IBMX on cdc39 strains were examined.

Figure 6.1 presents the results of this investigation. It is clear that the addition of 5mM IBMX was unable to prevent a significant decrease in the budding index of a cdc39 strain upon transfer to the restrictive temperature. The initial decrease in budding seen at 23°C, probably occurred as a result of "shock" following transfer from starter culture to test conditions. The 23°C + IBMX control indicated that IBMX was not toxic. Cells continued to grow and the budding index remained comparable with a 23°C culture lacking IBMX. Given the observation that catabolite repression interacts with both the pheromone response (Ruiz et al., 1984) and expression of the cdc39 mutation (Shuster, 1982b); and the indications that cdc39 and the mating system are closely involved (Reed, 1980; Shuster, 1982a; Connolly et al., 1983), it may be considered surprising that IBMX did not suppress cdc39. The results of Liao and Thorner (1981), upon which this work is based, have been questioned by Casperson et al. (1983). They failed to demonstrate any effects of alpha-factor on adenylate cyclase, implying that alpha-factor effects are not modulated via cAMP. If this is the case, it is difficult to understand how inhibitors of phosphodiesterase can antagonise alpha-factor action. Alternatively, failure of IBMX to suppress cdc39 may be because suppression of cdc39 is more stringent than suppression of alpha-factor arrest.

**FIGURE 6.1**      EFFECTS OF IBMX

Saccharomyces cerevisiae 2-15 was grown at 23°C in YMM supplemented with adenine. At  $3 \times 10^6$  cells/ml the cells were harvested and resuspended in YMM+ adenine buffered with 50mM sodium succinate (pH 5) at  $1 \times 10^6$  cells/ml. The sample was split into 4 fractions; 2 were incubated at 23°C (□ ■) and 2 at 36°C (○ ●). 5mM isobutylmethylxanthine (IBMX) was added to the samples indicated by filled symbols. At the times indicated, samples were taken, sonicated for 10 seconds and fixed in 4% formaldehyde. Percentage buddedness was determined by counting a minimum of 500 cells per sample. Figures presented indicate the change in the percentage of budded cells relative to an initial value of 55%.



## STARVATION OF *bcy1* CELLS

### INTRODUCTION

*bcy1* is a mutation that leads to high levels of cAMP independent protein kinase activity. It suppresses the requirement for cAMP of a mutant lacking functional adenylate cyclase (*cyr1*) (Matsumoto *et al.*, 1982a). In the absence of cAMP, *cyr1* mutants arrest in G1 as unbudded cells. Reciprocal shift experiments have demonstrated that this is at or near the point of nutritional arrest (Matsumoto *et al.*, 1983a). These results form part of the evidence indicating that cAMP is involved in signalling nutritional status (Chapter 1). Although starvation for any essential nutrient leads to G1 arrest (Johnston *et al.*, 1977), the evidence discussed in Chapter 1 suggests that cAMP plays a more significant role in the response to nitrogen starvation than to other nutrients. Following experiments in which *bcy1* cells were starved for nitrogen, Matsumoto *et al.* (1983a) concluded that; "These results suggest that the *bcy1* mutation suppresses G1 arrest caused by nutritional limitation." This statement was examined further by comparing the responses of *bcy1* and wild-type cells to nitrogen and/or glucose starvation.

### RESULTS AND DISCUSSION

Matsumoto *et al.* (1983a), have indicated that nitrogen starvation of *bcy1* cells leads to random arrest and decreased cell viability. They explained both these features by saying "The *bcy1* cells undergo one more cycle of division in the absence of growth and consequently reduce cell viability". As a reason for inviability this explanation is difficult to reconcile with the

results of Matsumoto et al. They indicated that over 48 hours, homozygous bcy1 diploids and homozygous cyr1/bcy1 diploids increased in cell number by a factor of approximately 1.7. During this period viability dropped to around 10%. The wild-type control underwent a 2.46 increase in cell number yet their viability is quoted at 242%!

Figure 6.2 indicates the proliferation of wild-type (panel A) and bcy1 (panel B) cells on various media. Since the figure plots the increase in cell number as measured by a particle counter, it gives no indication of mass increase. In both cases cells grown on complete media proliferate well. The absence of glucose and nitrogen prevents the proliferation of both strains. The patterns given by the absence of nitrogen or glucose show little difference between the wild-type and the mutant. It is clear that the bcy1 strain does not significantly increase in cell number in response to nutrient starvation.

Matsumoto et al. (1983a), have demonstrated that the budding-index (% of population with buds) of wild-type cells fell from 79% to 10% in response to nitrogen starvation. In bcy1 strains it fell from around 85% to 73%. Figure 6.3 illustrates the effect of starvation on the budding index of bcy1 and wild-type strains. As expected, cells grown in complete media (Figure 6.3A) retained a high budding index for many hours. The budding index in both populations began to fall as nutrients were exhausted. It can be seen that the bcy1 population retained a higher budding index. When starved of nitrogen (Figure 6.3B), both populations exhibited a fall in their budding index with



FIGURE 6.2      THE GROWTH OF bcy1 AND A WILD-TYPE STRAIN

The wild-type strain AM1-9C (panel A) and bcy1 strain AMI80-2B (panel B) were grown in YMM + histidine and adenine at 23°C. At t=0 the cells were collected, washed and resuspended at  $2 \times 10^6$  cells/ml in fresh media.

( ● )    0.67% YNB + 4% glucose, adenine and histidine.

( □ )    4% glucose + adenine and histidine.

( ■ )    0.67% YNB + adenine and histidine.

( ○ )    adenine and histidine.

Samples were taken at the indicated times and cell number determined in an electronic particle counter.

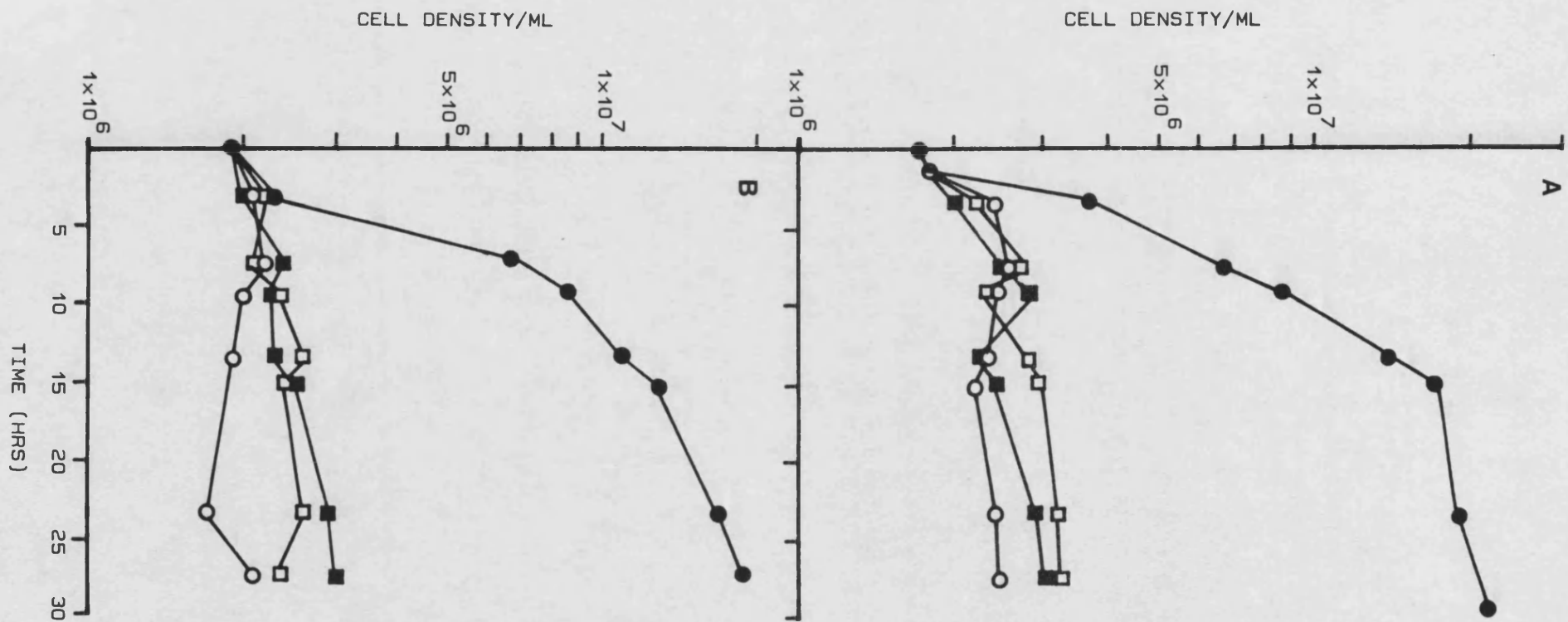


FIGURE 6.3      EFFECT OF STARVATION ON THE BUDDING INDEX

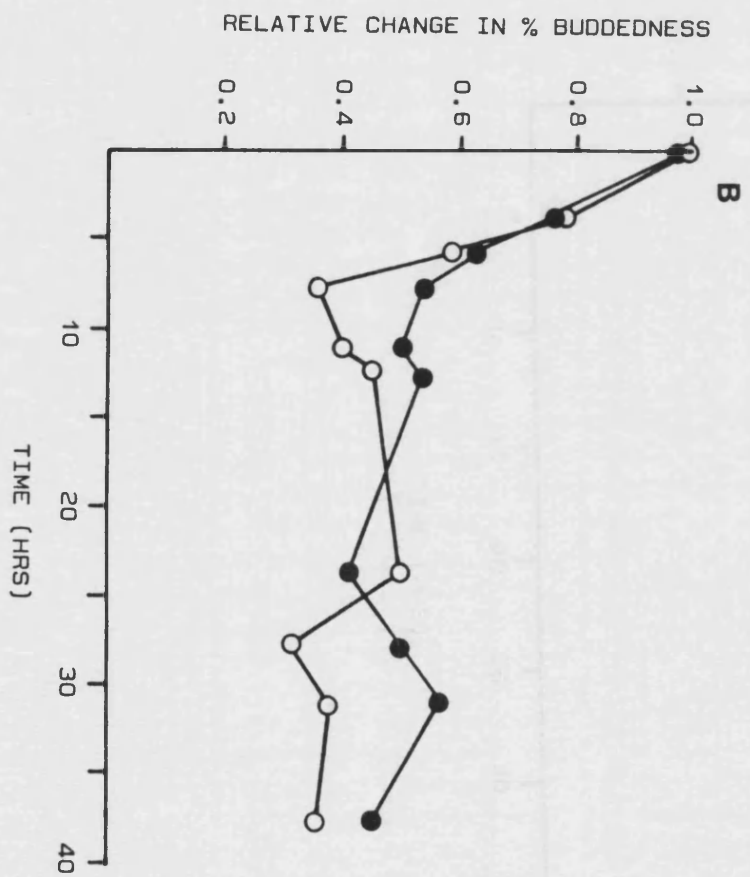
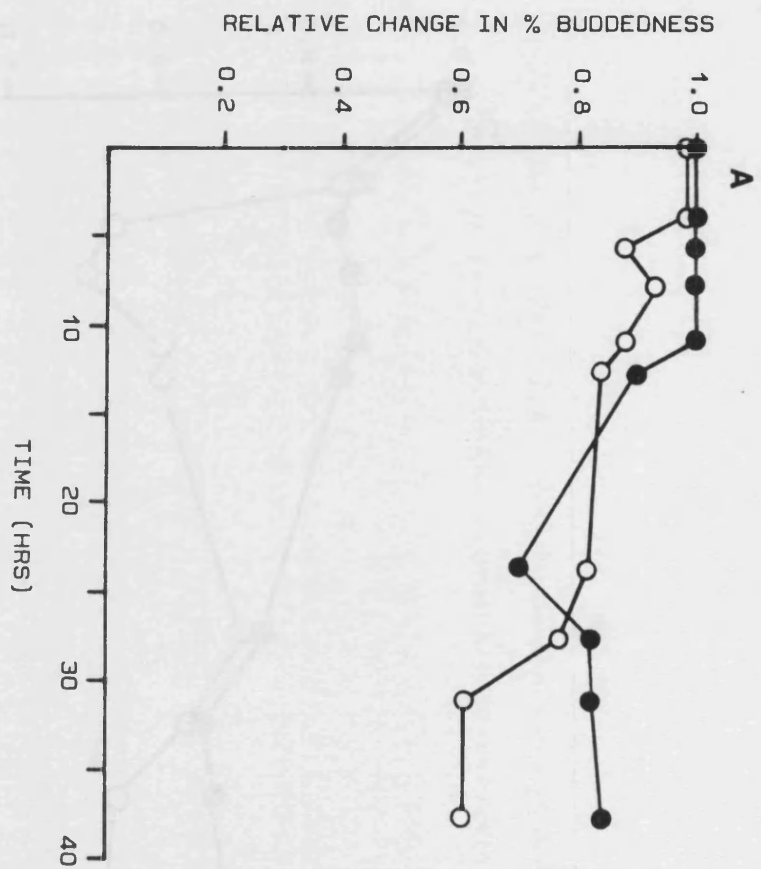
The wild-type strain AM1-9C ( O ) and bcy1 strain AM180-2B ( ● ) were grown in YMM + histidine and adenine at 23°C. At t=0 the cells were collected, washed and resuspended at  $2 \times 10^6$  cells/ml in fresh media.

A    YMM + adenine and histidine.

B    4% glucose + adenine and histidine.

C    0.67% YNB + adenine and histidine.

At the times indicated, samples were taken, sonicated for 10 seconds and fixed in 4% formaldehyde. Percentage buddedness was determined by counting a minimum of 500 cells per sample. The ordinate axis expresses the change in the number of cells containing small buds ( half mother cell size) relative to the time zero values. (AM180-2B=43%, AM1-9C=40%)



similar kinetics. Starvation for glucose resulted in a situation in which bcy1 cells constantly exhibited higher budding indices than the wild-type control (6.3C).

There are three possible reasons why these results differ from those of Matsumoto et al., (1983a). 1) They used diploid cells in their study; haploid cells were used in these experiments. 2) Because a rich nutrient media was used for starter cultures, the budding indices in the experiments of Matsumoto et al. started around the 80% level. By using supplemented yeast minimal media for the starter cultures the initial levels in these experiments were around 45%. This gave Matsumoto et al. a much greater resolution for the expression of any differences. Nevertheless, differences of the order described should have been obvious, even using our low resolution system. 3) The budding index of the wild-type used by Matsumoto et al., (1983a) fell rapidly upon transfer to nitrogen free media. The wild-type control used in our experiment retained a high budding index under conditions in which it may have been expected to fall much lower. Although not affecting the absolute budding index, a control in which budding fell rapidly to around 5% would have made the bcy1 strain appear to remain relatively budded.

Figure 6.4 presents the results of nutrient starvation on viability. Total cell counts were determined using a particle counter. Although this instrument is adequate for relative counts, absolute counts can be unreliable. Consequently, discrepancies between viable counts and particle counts

FIGURE 6.4 VIABILITY OF bcy1 STRAINS

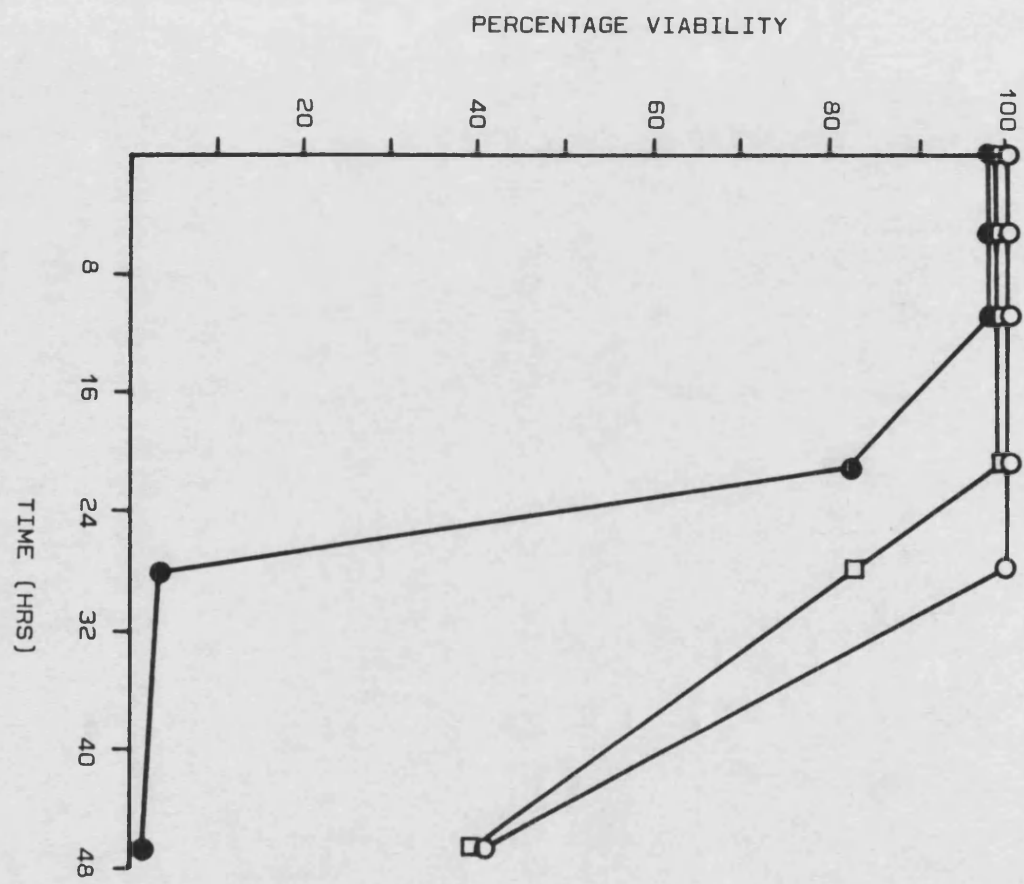
AM180-2B (bcy1) was grown to mid-exponential phase in YMM + histidine and adenine at 23°C. At t=0 cells were harvested, washed and resuspended at  $2 \times 10^6$  cells/ml in fresh media.

( O ) 0.67% YNB, 4% glucose + adenine and histidine.

( □ ) 0.67% YNB + adenine and histidine.

( ● ) 4% glucose + adenine and histidine.

At the indicated times samples were taken, counted in an electronic particle counter, and plated onto YEPD. % viability was calculated as (total cells/ml / cells capable of forming colonies/ml).



occasionally indicated greater than 100% viability. When this occurred, the actual percentage viability was recorded as 100%.

The curve describing the effect of nitrogen deprivation is similar to that presented by Matsumoto et al., (1983a). Starvation for nitrogen results in a rapid decline in viability. Although starvation for glucose also leads to reduced viability, the rate at which viability falls is slower than for nitrogen. Since starvation for either glucose or nitrogen results in decreased viability, it is inevitable that the viability of bcy1 strains growing on complete media would decline as the available nutrients are consumed.

Matsumoto et al. (1983a) report that "bcy1 cells sometimes continued bud emergence for multiple cycles without further nuclear division". This property was seen on several occasions when working with bcy1 strains. Although several attempts were made to characterise this phenotype, little success was achieved.

Failure was mainly due to the apparently random appearance of this phenotype. Nevertheless, it was possible to induce the phenotype by incubation in presporulation media for three days. Figure 6.5 presents two different bcy1 strains, the multiply budded phenotype described by Matsumoto et al. is evident in both cultures. Figure 6.6 illustrates two different wild-type strains. Both gave the unbudded G1 arrest typical of cells responding to nutrient starvation.

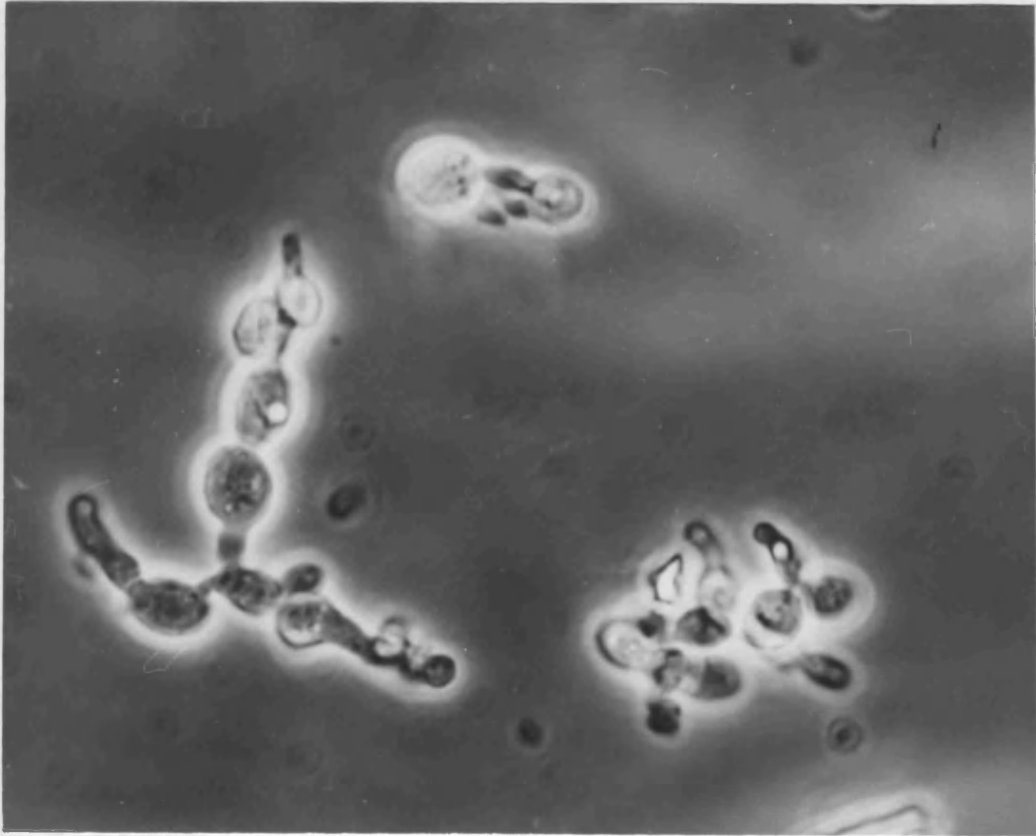
This section sought to indicate whether bcy1 mutants had an altered response to starvation in general or to nitrogen deficiency in particular. From the results presented it is clear



FIGURE 6.5      PHENOTYPE OF bcy1 CELLS

AM9-10A (bcy1, cyr1) (photograph A) and AM180-2B (bcy1) (photograph B) were grown for 3 days in yeast presporulation media. Samples were taken, sonicated for 10 seconds and viewed through a x40 phase contrast objective. Total magnification = 400x

A



B

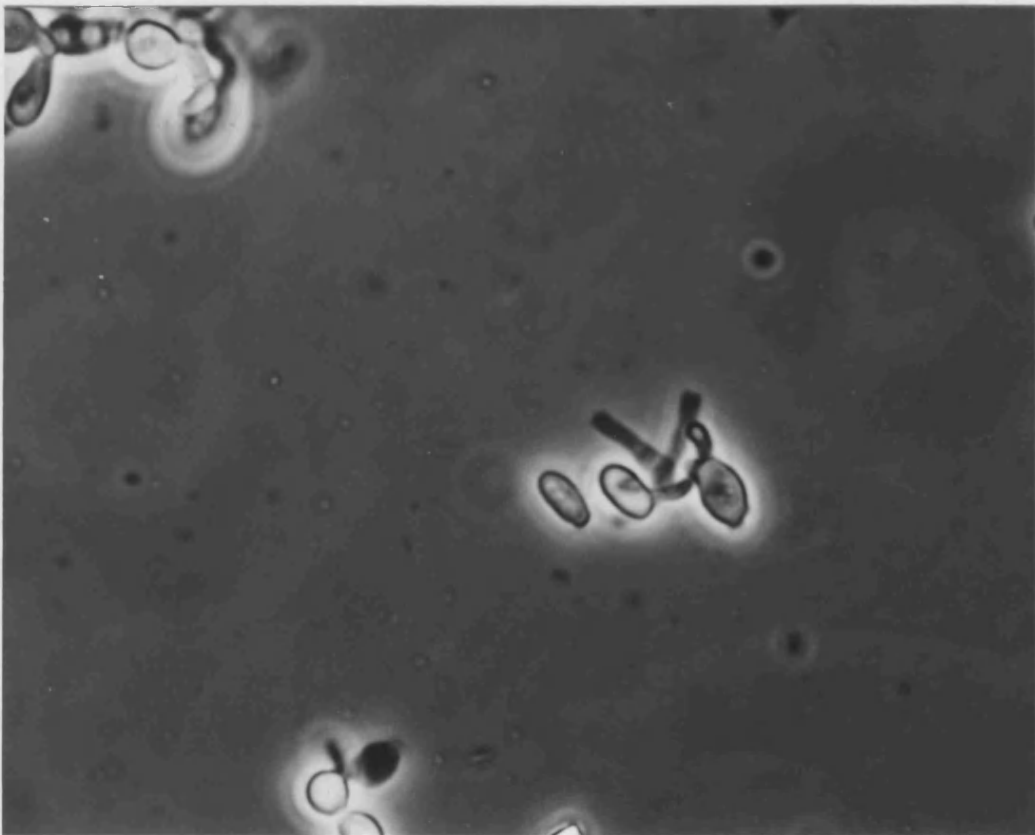
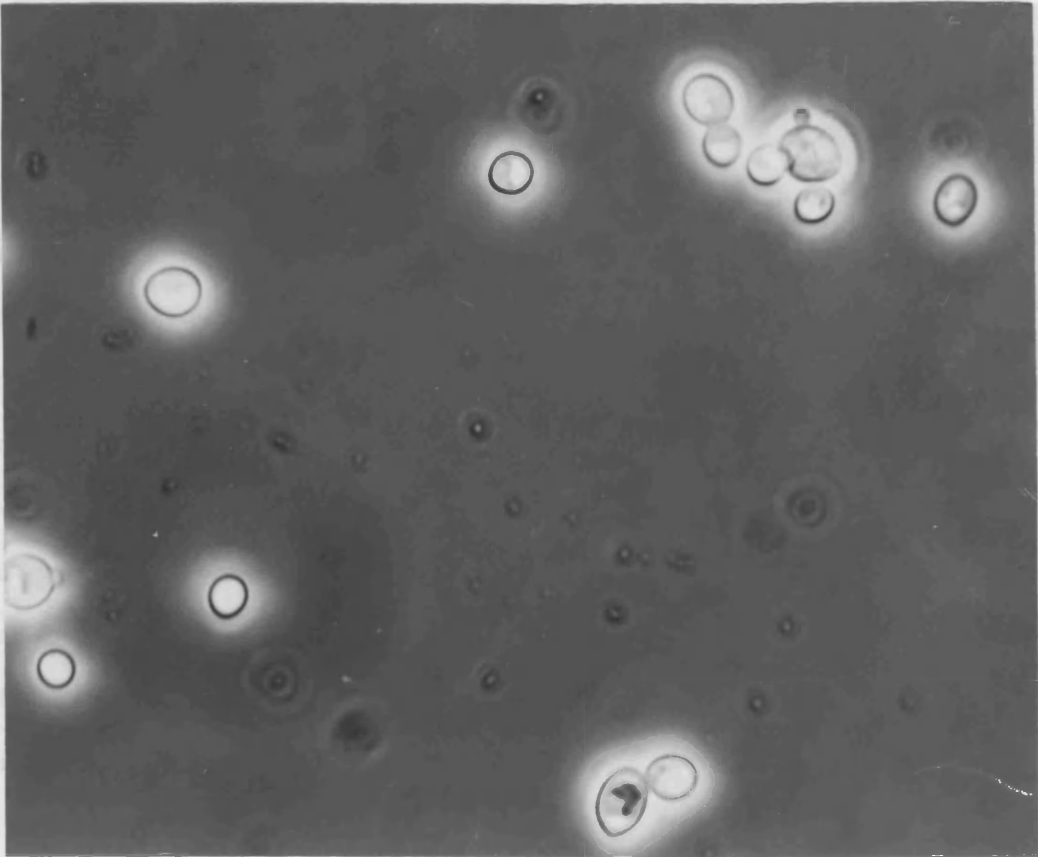


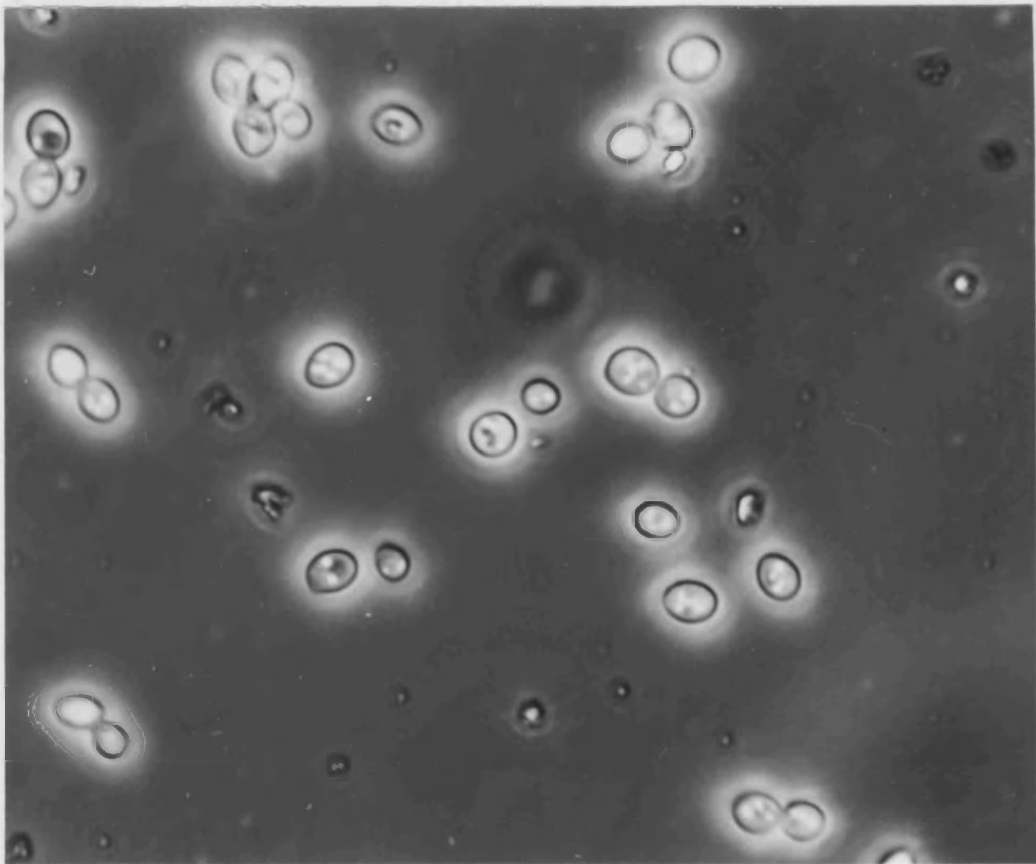
FIGURE 6.6      PHENOTYPE OF BCY1 CELLS

AM3-3D (photograph A) and AM3-4B (photograph B) were grown for 3 days in yeast presporulation media. Samples were taken, sonicated for 10 seconds and viewed through the x400 phase contrast objective.

A



B



that neither a bcy1 nor a wild-type strain are capable of sustaining prolonged proliferation in the absence of a carbon or nitrogen source. Both strains responded more strongly and rapidly to nitrogen starvation than to glucose starvation. Although the bcy1 strain consistently retained a higher budding index, the significance of this is not clear. Starvation for nitrogen led to a more rapid inviability than starvation for glucose. It is possible that this reflects a genuine difference in the response of bcy1 cells to starvation for different nutrients. Alternatively, the slower budding index response of both wild-type and bcy1 strains to starvation for glucose, may indicate that all cells elicit a "starvation signal" much more quickly under conditions of nitrogen starvation than carbon starvation. In this case, removal of nitrogen leads to faster inviability than the removal of carbon simply because the cells starve faster. If this is so, bcy1 cells do indeed respond to "starvation" rather than to the absence of nitrogen alone.

INTERACTION OF ste8 AND ste9 WITH cdc39INTRODUCTION

As discussed in chapter 3, heterozygosity at the mating type locus suppresses the G1 arrest of cdc36 and cdc39 mutants (Reed, 1980). A follow-up study quantified this observation and demonstrated that MATa/MAT $\alpha$  diploids arrested randomly at a temperature 2 degrees above the restrictive temperature for haploid cdc39 strains (Shuster, 1982a). Connolly *et al.* (1983) isolated mating pheromone resistant mutants in a "start" mutant background, and demonstrated that all pheromone resistant cdc36 or cdc39 strains failed to arrest homogeneously at the restrictive temperature. They concluded that G1 arrest of cdc36 and cdc39 strains is dependent upon an intact mating system. Since resistance to alpha-factor may be caused by several mechanisms, it is surprising that all resistant strains showed random arrest. It is difficult to understand why strains becoming resistant to alpha-factor by losing the alpha-factor receptor, or switching their mating type should confer this property. Nevertheless, these results "suggest that the CDC36 and CDC39 gene products are involved directly in the regulation of cell division mediated by mating factors" (Shuster, 1982a). If these genes are involved in the mechanism by which cells transduce the mating pheromone response, one would predict possible interactions between cdc36 and cdc39 with mutants defective in pheromone response.

This prediction was partly investigated by Shuster (1982a), who examined interactions between start mutants cdc28, cdc36,

cdc37 and cdc39, and sterile mutants ste4, ste5, ste7, ste11 and ste12. Sterile mutants (ste) are strains that have significantly reduced mating ability (Hartwell, 1980). It was demonstrated that cdc36 and cdc39 could suppress ste4 and ste5 in an allele specific manner, no other interactions were found. Subsequently, suppression of ste5 by certain alleles of cdc28 was demonstrated (Reed, 1984). It was suggested that the marked allele specificity of interactions between cdc36 and cdc39, and ste4 and ste5 implied physical or functional interactions between these 4 genes (Reed, 1984). Although this study described the suppression of ste mutants by cdc mutations, it did not address the reciprocal suppression of cdc mutants by ste mutants. The only information pertaining to this was provided by Hartwell (1980), who demonstrated that none of the steriles would suppress cdc28.

The suggestion that cdc36 and cdc39 operate as part of the mating response mechanism may be supported by continuing the study of interactions between cdc and ste mutants. Since mating type heterozygosity suppresses cdc36 and cdc39, a logical prediction is that mutations mimicking heterozygosity may also suppress them. ste8 and ste9 are mutations that confer resistance to alpha factor (Hartwell, 1980). When characterised they were found to have much reduced mating efficiencies (ste8=  $1 \times 10^{-6}$ , ste9=  $2 \times 10^{-4}$ ). They were no longer inducible for agglutination, produced less than 2% wild type levels of mating pheromone, and had less than 3% of the parental mating pheromone destruction index. Haploid controls showed approximately 3%

polar budding, with a diploid value around 73%. ste8 haploids showed 55% polar budding, a ste9 strain had a value of 63%. The behaviour described is more typical of diploid cells than haploid cells. Indeed, ste8 and ste9 are indistinguishable from MATa/MAT $\alpha$  diploids. They have subsequently been demonstrated to be allelic to sir3 and sir4 (Hartwell, 1980). These mutations allow expression of the silent MAT alleles at HMa and HM $\alpha$  (Rine, 1979). Described below is a preliminary investigation into the interactions of cdc39 with ste8 and ste9.

## RESULTS AND DISCUSSION

### MAT heterozygosity

As a control the effects of temperature on MAT $\alpha$  and MATa/MAT $\alpha$  diploids was examined. The results presented in Figure 6.7 and 6.8 are in good agreement with those of Shuster (1982a). The increase in cell density of each population was more or less affected by transference to the restrictive temperature. The diploid SR665/2-15 has a delayed response to the restrictive temperature and a slow but steady increase in cell number continued throughout the experiment. The budding index of the cdc39/cdc39 diploid remains high, while that of the cdc39 haploid drops rapidly after the shift to 36°C. Although the effects on mass were not determined in this study, Shuster (1982a) has pointed out that the increase in cell number and heterogeneous arrest occurring as a result of mating-type heterozygosity is not matched by an increase in mass. These results suggest that MAT heterozygosity is capable of suppressing the cell cycle defect of



FIGURE 6.7      CARBON SOURCE EFFECTS

SR665-1, a cdc39 haploid ( O ) and SR665/2-15, a homozygous cdc39 diploid ( ● ) were grown at 23°C in YEPD. At t=1 hour the cultures were shifted to 36°C. Samples were taken at the indicated times and cell numbers assayed in an electronic particle counter. The ordinate scale plots normalised cell number (normalised as in Figure 3.1). Initial cell density for SR665-1=  $1.1 \times 10^6$ . Initial density for SR665/2-15=  $3.4 \times 10^6$ .

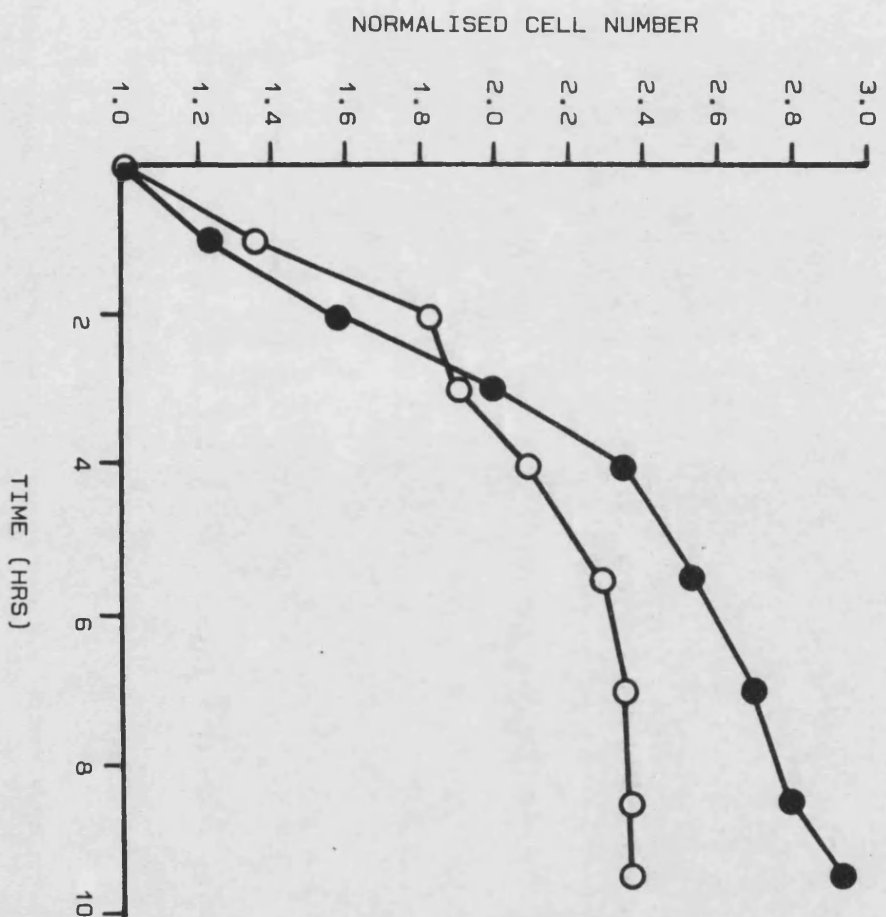
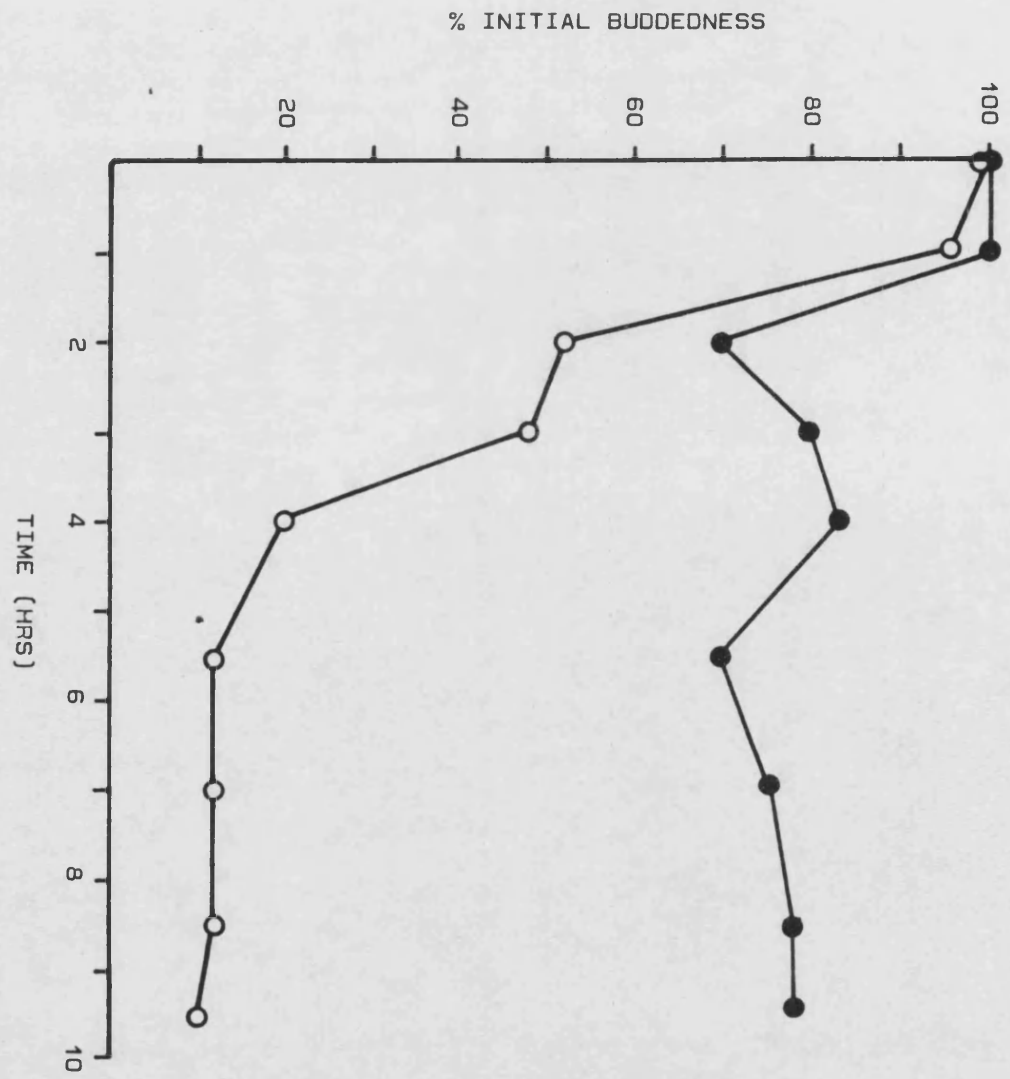


FIGURE 6.8 SUPPRESSION OF G1 ARREST BY MATING-TYPE  
HETEROZYGOSITY

SR665-1, a cdc39 haploid ( O ), and SR665/2-15, a homozygous cdc39 diploid ( ● ) were grown at 23°C in YEPD. At t=1 hour the cultures were shifted to 36°C. Determinations of percentage buddedness are based on a minimum of 500 cells per sample. Samples, taken at the indicated times, were sonicated for 10 seconds and fixed in 4% formaldehyde. Initial buddedness for SR665-1= 48%. Initial buddedness for SR665/2-15= 56%.



cdc39 but not the growth defect (see Chapter 1).

#### Interaction with ste8 and ste9

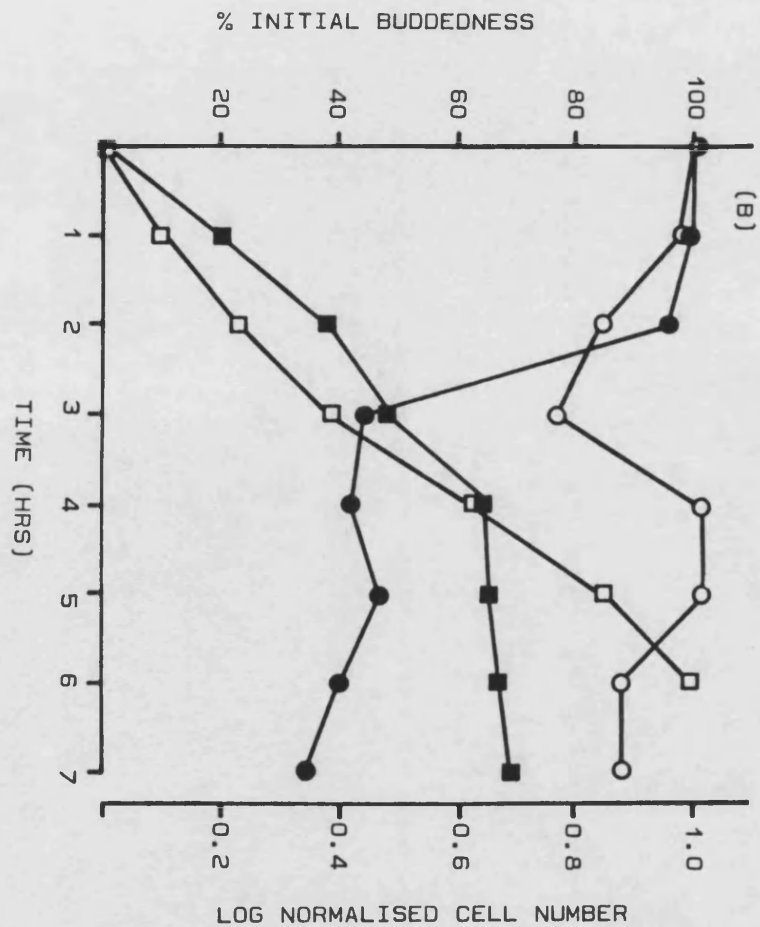
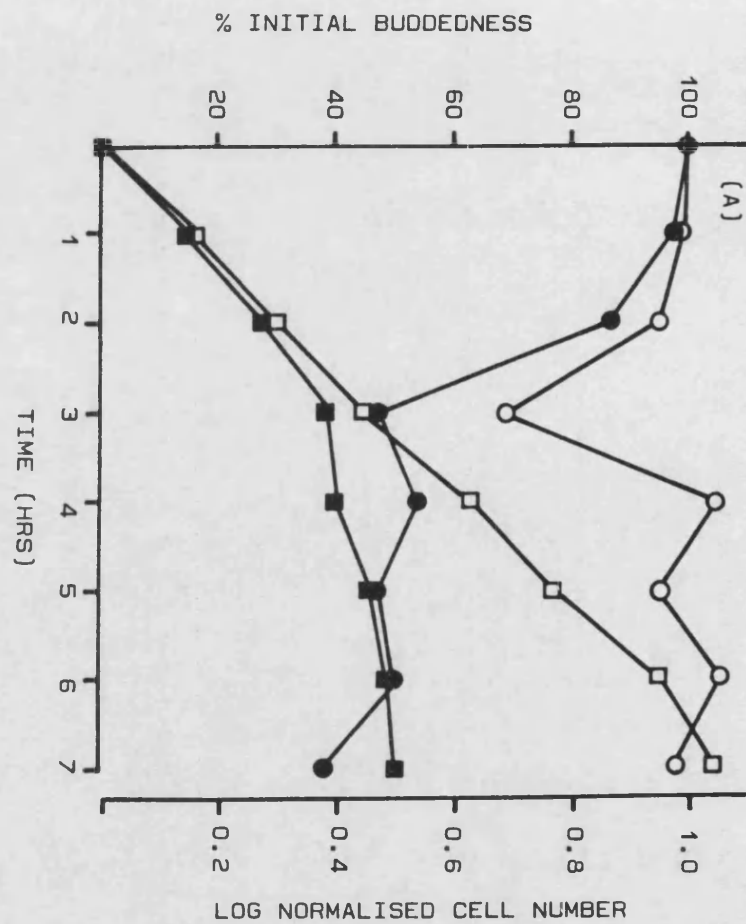
SR665-1 (cdc39) was crossed with 381G (wild-type), 381G-91A (ste8-5), and 381G-62C (ste9-1). After selecting diploids, they were sporulated and subject to tetrad analysis. In each case a minimum of 10 asci were dissected and all showed a clear 2:2 segregation of temperature resistance to temperature sensitivity.

It was clear that neither ste8 nor ste9 were capable of suppressing the growth defect of cdc39 as scored on agar plates. Since mating-type heterozygosity does not allow an accumulation of mass in cdc39 strains, suppression at this level was not expected. Considering this, a single tetrad from the 381G-62C cross (SR665/62(21)) was subject to more quantitative tests. The pattern of growth and the budding index of these cells were followed in shake flask cultures growing in YEPD at 36°C (Figure 6.9). Haploids SR665/62C(21B and 21C) continued to proliferate after the shift to 36°C. The budding index of these cells remained around the initial level. Strains SR665/62C(21A and 21D) exhibited a growth pattern typical of temperature sensitive cdc39 strains. In both cases the budding index of the cells fell rapidly as proliferation stopped. These results indicate no obvious suppressive effects of ste9 on cdc39.

Quantitative tests on cells from the SR665-1 x 381G-91A cross were restricted to the two temperature sensitive spores from 2 asci (SR665/91A(1) and SR665/91A(2)), since suppression of the cell cycle defect of cdc39 will only be seen in cells showing a temperature sensitive phenotype. In both cases one of the

FIGURE 6.9      EFFECT OF TEMPERATURE ON THE GROWTH AND BUDDING INDEX OF CELLS FROM A cdc39 x ste9 ASCUS

SR665-1 (cdc39) was crossed with 381G-62C (ste9). The resulting diploid (SR665/62C) was sporulated and the progeny from a single ascus examined further. At t= 2 hours cells were shifted from 23°C to 36°C. Panel A describes the growth ( □ ■ ) and budding index ( ○ ● ) of SR665/62C(21A) (full symbols, initial budding (IB) =48%) and SR665/62C(21B) (open symbols, IB =53%). Panel B describes SR665/62C(21C) (open symbols, IB =52%), and SR665/62C(21D) (closed symbols, IB =47%). Percentage buddedness determined as in Figure 6.5.



temperature sensitive strains retained a high budding index while that of the other fell rapidly. Figure 6.10 presents the data regarding SR665/91A(1). Figure 6.11 confirms that both these strains are temperature sensitive for growth at 36°C. Comparison of Figures 6.10 and 6.8 indicate that the kinetics of suppression by ste8 are very similar to that caused by MAT heterozygosity. The transient fall in the percentage of budded cells exhibited by SR665/2-15 (Figure 6.8) and SR665/91A(1C) (Figure 6.10), can be explained as a response to the "shock" of the temperature increase. This feature is also seen in cells that are temperature resistant (SR665/62C(21B & 21C), Figure 6.9).

To correlate these observations with the presence of the ste8 gene, quantitative mating experiments were performed using the following procedure (taken from Hartwell, 1980).

- 1) To ensure that the observed differences in mating efficiencies were due mainly to the test strains, the strains against which they were crossed were selected to be as genetically similar as conveniently possible.
- 2) The mating-type and nutrient requirements of the segregants from ascus SR665/91A(1) were established. These strains, along with appropriate controls, were then crossed to suitable partners.
- 3) Since two of the segregants contained a cdc39 gene, all were grown to about  $7 \times 10^6$  cells/ml in YEPD at 23°C. For tests at the restrictive temperature, one flask of each strain was shifted to 34°C for 4 hours.
- 4)  $2 \times 10^6$  cells from each culture and temperature were collected



FIGURE 6.10      EFFECT OF TEMPERATURE ON THE BUDDING INDEX OF  
CELLS FROM A cdc39 x ste8 ASCUS

SR665-1 (cdc39) was crossed with 381G-91A (ste8). The resulting diploid (SR665/91A) was sporulated and subjected to tetrad analysis. The two temperature sensitive progeny from a single ascus were examined further. Cells were grown at 23°C in YEPD, at t= 1 hour the populations were shifted to 36°C. Budding index was determined as in Figure 6.5. SR665/91A(1:C) = closed symbol, initial budding (IB)= 48%. SR665/91A(1:D) = open symbol, IB = 49%.

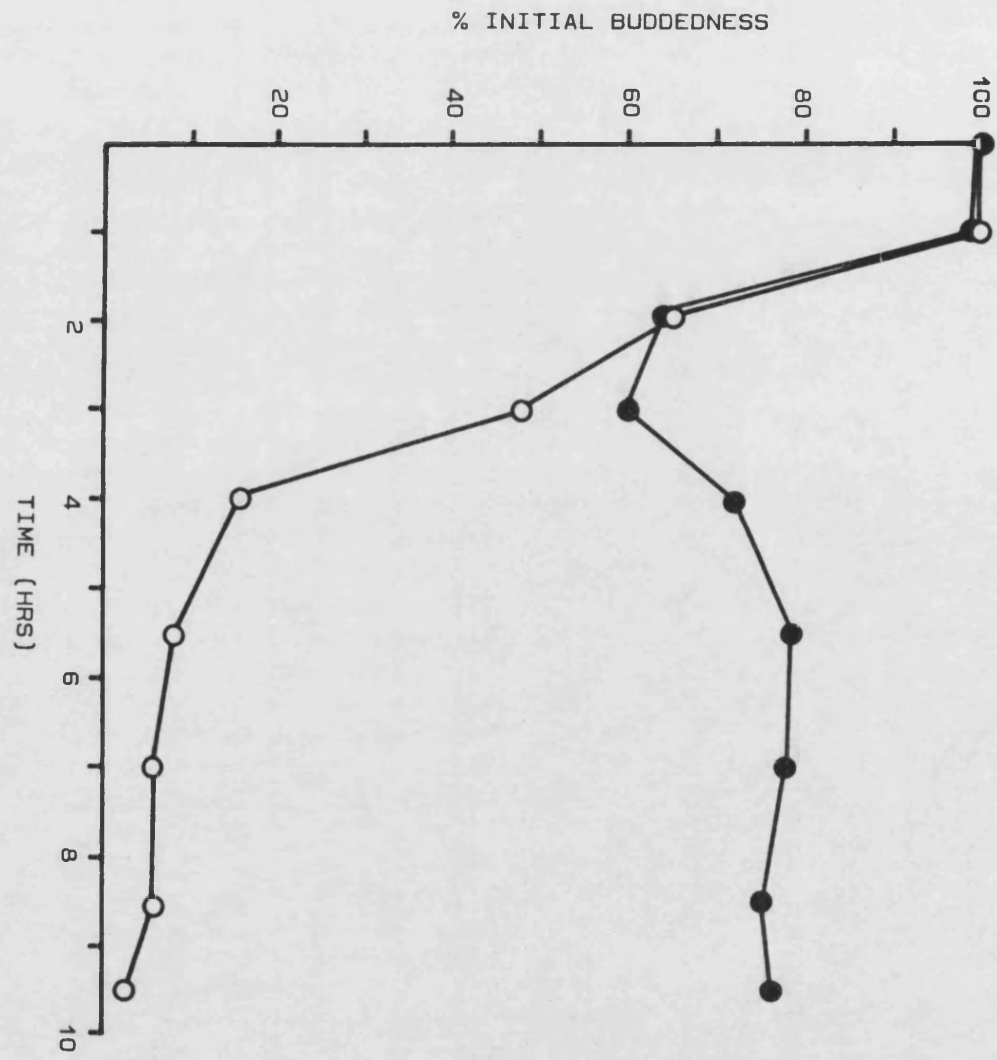
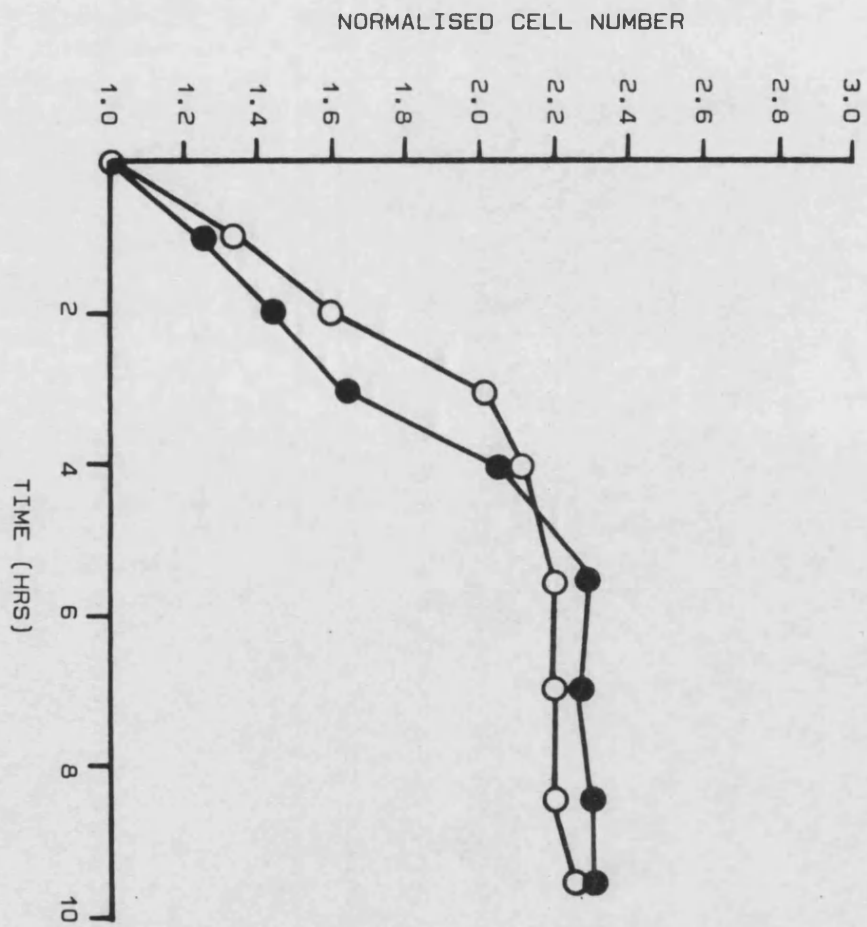


FIGURE 6.11      EFFECT OF TEMPERATURE ON THE PROLIFERATION OF  
CELLS FROM A cdc39 x ste8 ASCUS

SR665-1 (cdc39) was crossed with 381G-91A (ste8). The resulting diploid (SR665/91A) was sporulated and subjected to tetrad analysis. The two temperature sensitive progeny from a single ascus were examined further. Cells were grown at 23°C in YEPD, at t= 1 hour the populations were shifted to 36°C. Cell number was determined using a particle counter. The ordinate scale indicates normalised cell counts (normalised as in Figure 3.1). Initial density for SR665/91A(1C) ( ● ) =  $2.4 \times 10^6$  cells/ml. Initial cell density for SR665/91a(1D) ( ○ ) =  $3.8 \times 10^6$  cells/ml.



on a nitrocellulose filter.  $2 \times 10^6$  cells of a strain of the opposite mating type were collected on top.

5) Filters were then transferred to YEPD plates and incubated for 5 hours at  $23^\circ\text{C}$  or  $34^\circ\text{C}$ .

6) Each mating mix was resuspended in 5mls of yeast nitrogen base solution and sonicated for 10 seconds.

7) Following serial dilution, the cell suspension was plated onto selective media and YEPD.

8) Plates were then incubated at  $23^\circ\text{C}$ .

The results of this experiment are presented in Table 6. 1. They indicate that the ste8 and ste9 controls mate at a significantly reduced frequencies. Strains SR665/91A(1A & 1C) also show reduced mating frequencies and it was concluded that they contain a ste gene. Since SR665/91A(1C) was also the strain that retained a high budding index (Figure 6.10), it was tempting to conclude that the observed suppression of the G1 arrest of cdc39, is caused by the ste8 mutation. Failure to see suppression using ste9 can be explained in several ways and leads to several conclusions. A) ste9 and cdc39 are linked. Tight linkage would prevent the generation of cdc39/ste9 recombinant haploids. The lack of information concerning the map location of ste9 (sir4) means that this possibility cannot be excluded. B) Since suppression was examined in a single tetrad, it is possible that the effect is MAT specific. However, Hartwell (1980) reported that those properties of ste9 that were examined, were mating-type independent. The suppression of cdc39 by ste8 was seen in spores of both mating types (SR665/91A(1C) = MAT $\alpha$ ,

STRAIN	23°C		34°C	
	DIPLOIDS FORMED	MATING EFFICIENCY	DIPLOIDS FORMED	MATING EFFICIENCY
381G (WT)	$4.8 \times 10^6$	1	$2.3 \times 10^7$	1
381G-91A ( <u>ste8</u> )	$3.7 \times 10^5$	0.08	20	$8.7 \times 10^{-7}$
381G-62C ( <u>ste9</u> )	$2.5 \times 10^6$	0.53	20	$8.7 \times 10^{-7}$
SR665/91A(1 A)	$1.3 \times 10^6$	0.27	20	$8.7 \times 10^{-7}$
SR665/91A(1 B)	$1.0 \times 10^6$	0.21	$1.7 \times 10^6$	0.35
SR665/91A(1 C)	$1.1 \times 10^6$	0.23	50	$1 \times 10^{-5}$
SR665/91A(1 D)	$1.2 \times 10^6$	0.25	$2.4 \times 10^6$	0.42

TABLE 6.1      MATING EFFICIENCIES

SR665-1 (cdc39) was crossed with 381G-91A (ste8), the resulting diploid was sporulated and a single ascus (SR665/91A(1)) examined for the segregation of the ste8 gene. The sterile phenotype was determined using the quantitative mating assay described in the text. Mating efficiency is expressed as (diploids formed / diploids formed by 381G (WT) at the same temperature).

SR665/91A(2D)= MATa) and would tend to support the observation of Hartwell that the effects of both ste8 and ste9 are mating-type independent. C) Failure of 381G-62C to suppress eliminates the possibility that an unknown gene in the 381G background is responsible for the observed results.

Although Figure 6.10 suggests that ste8 suppression is analogous to that of MAT heterozygosity, Figure 6.11 illustrates a difference of unknown significance. Whilst suppression of cdc39 by mating-type heterozygosity allows continued proliferation, suppression by ste8 does not. The implication is that while ste8 causes first cycle random arrest, MAT heterozygosity allows several rounds of division. Shuster (1982a) points out that although a MATa/MATa, cdc36/cdc36 diploid showed the same increase in mass as a MATa/MAT $\alpha$ , cdc36/cdc36 diploid (2 fold), the MAT heterozygous diploid showed twice the cell number increase. From his figures the increase in cell number of a cdc39 strain was even more pronounced (4 fold). These observations suggest that as MATa/MAT $\alpha$ , cdc39/cdc39 diploids continue proliferation at the restrictive temperature, their cell mass becomes much reduced. Although not identical, this situation is reminiscent of the behaviour exhibited by whi1 (Carter and Sudbery, 1980) and bcy1 (Matsumoto et al., 1983a) cells, when challenged with nutrient starvation. One could speculate that this illustrates a complexity of "start" that is over simplified by the constraints of strictly defined classes of "start" mutant.

CHAPTER 7  
DISCUSSION

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### DISCUSSION

The work discussed in this thesis has concentrated on the regulatory role of the CDC39 gene in the cell cycle of yeast. As yet both the nature and function of the CDC39 gene product remain unknown. However, the circumstantial evidence available allows speculative conclusions to be drawn. As with all models those devised to explain the role of CDC39 should accommodate all the available evidence. This evidence falls into two groups each suggesting different functions for CDC39.

Linking CDC39 with the mating pheromone response system are several lines of evidence. A) Cells containing the mutant cdc39 gene arrest in G1 at a point interdependent with mating pheromone (Reed 1980). The growing, shmooing phenotype of arrested cdc39 cells is similar to that of cells arrested by mating pheromone. B) Heterozygosity at the mating-type loci suppresses the G1 arrest of homozygous cdc39 diploids (Chapter 6). C) ste8, a mutation that leads to expression of silent mating-type loci giving a diploid like phenotype (Hartwell 1980), may suppress cdc39 (Chapter 6). D) cdc39 is capable of suppressing the sterile phenotype of strains containing the ste4 and ste5 mutations (Shuster 1982a).

A role for CDC39 in the regulation of growth in response to the carbon source is suggested by the following observations. A) The G1 arrest of cdc39 cells is suppressed by derepressive carbon sources (Chapter 3). B) hex1, a mutation in hexokinase PII (Entian and Frohlich 1984), leads to the constitutive

derepression of several enzymes normally under the regulation of catabolite repression. Evidence suggests that hex1 is capable of suppressing cdc39.

At first sight the mating system and carbon metabolism would seem to be unrelated. However, besides both interacting with cdc39, carbon source effects on the sensitivity of cells to mating pheromone (Ruiz et al., 1984) indicate a more direct relationship.

The model that best fits these data was suggested by Shuster (1982a). It has been expanded to explain carbon source and mating-type heterozygosity effects and is presented as Figure 7.1. The basis of this model is that the CDC39 gene product has two regulatory functions. At intermediate gene product levels it serves to promote growth. At higher levels it prevents the proliferation inhibitory effects of an intact mating system. The model calls for CDC39 to act as an inhibitor of a constitutively active mating system. The addition of mating pheromone to CDC39 cells reduces the activity of the gene product to a level insufficient to prevent inhibition by the mating system but high enough to maintain promotion of growth. When cdc39 mutants are transferred to the restrictive temperature, the activity of the functional CDC39 protein falls slowly. A slow decline explains the continued growth of cdc39 strains in the absence of proliferation. Since proliferation is actively inhibited by an intact mating system, cells lacking an intact system (MATa/MAT $\alpha$ , and ste8) continue to proliferate in the absence of sufficient CDC39 gene product.

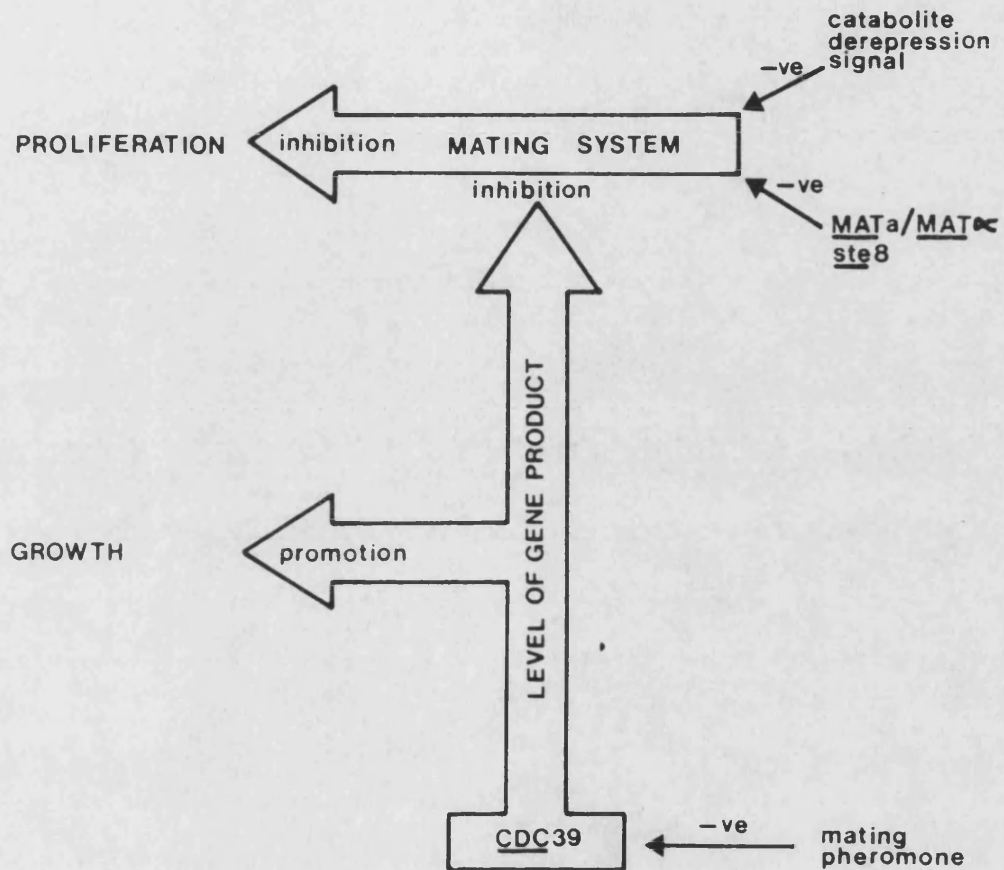


FIGURE 7.1      MODEL OF CDC39 ACTION

A schema offered to explain elements of data relating carbon metabolism and mating pheromone response to the regulation of growth and proliferation by CDC39.  
 -ve indicates an inhibitory action.

By proposing that one aspect of catabolite derepression is to prevent the inhibition of proliferation by the mating system, the observed interactions between derepression and mating and between derepression and cdc39 can be explained. Since the proposed results of a derepression signal are additive to those functions of CDC39, that regulate proliferation but not growth, the level of the CDC39 product would need to fall further before the mating system becomes free to inhibit proliferation. Consequently, cells metabolising pyruvate are less sensitive to mating pheromone than glucose grown cells. Similarly, derepressed cdc39 cells are able to maintain sufficient "inhibition signal" to initiate a further round of cell division. Once the cdc39 gene product level falls below the growth promotion threshold these cells will cease growth and arrest at random throughout the cell cycle.

The model described suggests that proliferation and growth are dependent upon different levels of a single CDC39 function. Alternatively, it is conceivable that the CDC39 gene product is bifunctional and that the growth and proliferation defects are the results of two mutations in the same protein. However, the criteria used to select class I start mutants (Reed 1980; Chapter 1) demands continued growth in the absence of proliferation. Consequently, mutants defective in both domains would fail to grow and would have been rejected. The continued growth of cdc39 cells indicates that the gene product must temporarily retain partial activity. The selection procedure used would ensure that this class were selected.

The properties of cdc39 cells have prompted the suggestion that cdc39 is simply an element of the mating pheromone transduction pathway. However, the similarities between cdc39 and cdc28, a class I mutant not implicated in pheromone response, indicate that if this is the case cdc39 may have functions outside its putative role in mating. Although cdc28 is not suppressed by mating-type heterozygosity it does show an allele specific suppression by derepressive carbon sources (Chapter 3). cdc28 will not suppress ste4 but will, in an allele specific manner, suppress ste5. Clearly cdc28 and cdc39 share a subset of functions that relate to both mating and carbon metabolism.

If a cell containing cdc39 is a cell that "thinks" mating pheromone is present it could be considered to be an extreme supersensitive (sst). sst2-1 is described as an intrinsic defect that makes cells supersensitive to mating pheromone (Chan and Otte 1982b). It has been reported that, in the absence of mating pheromone, sst2-1 causes a growth debility. This mutation indicates that under appropriate conditions the mating system is capable of exerting effects on growth.

It should be understood that this discussion has concentrated on regulation by CDC39. Although cdc36 is phenotypically identical to cdc39 and is likely to function in a similar manner, the regulatory functions of other start genes probably operate via different mechanisms. "Start" defines the integration of these mechanisms serving to regulate cellular response to the various environmental effectors. The characterisation of cAMP metabolism mutants is rapidly

illuminating the role of cAMP and protein phosphorylation (Chapter 1), and is demonstrating the value of yeast as a model organism capable of generating results applicable to higher eukaryotes.

Figure 1.3 represents a summary of the mutations presently known to influence the cell cycle. Complete understanding of cell cycle regulation will have to explain the interactions implicit in this diagram. Although many questions remain to be answered the great effort to date has provided a frame upon which our understanding of cellular regulation is rapidly crystallising.

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